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On-line Trace Enrichment in High Performance Liquid Chromatography Using XAD-2 Precolumn for the Determination of Lonazolac in Human Plasma

Hye Suk Lee, Eun Joo Kim, Ok Pyo Zee and Yoon Joong Lee*
Korea Research Institute of Chemical Technology(KRICT), Daejeon 302-343, and
**College of Pharmacy, Sungkyunkwan University, Suweon 440-746, Korea*
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Abstract□A new column-switching high performance liquid chromatographic method was developed for the determination of lonazolac in plasma. This method was based on the on-line trace enrichment of lonazolac using a precolumn packed with Amberlite XAD-2. The analysis was complete in 20 min. between injectons and the limit of detection was 0.1 μg/m*l* using 100 μ*l* of plasma. The method was linear in range of 0.1-10 μg/m*l* with a correlation coefficient of 0.9991. Absolute recovery of lonazolac from the spiked plasma samples ranged from 95.6% to 97.1%. The method was shown to be reproducible over the concentration range studied.

Keywords□ Amberlite XAD-2, on-line trace enrichment, lonazolac, HPLC, plasma

Determination of drugs in complex biological fluids like plasma by high performance liquid chromatography (HPLC) frequently involves sample cleanup steps, such as protein precipitation, liquid-liquid extraction and solid phase extraction, prior to injection onto the chromatograph. These work-up preocedures are tedious to perform, prone to errors and often give rise to loss of sample from incomplete extraction or formation of artefacts.

An alternative to using adsorbent columns "off-line" is the "on-line" incorporation of them into the flow scheme of a liquid chromatograph as a precolumn in advance of the analytical column. In this way concentration and cleanup of complex biological samples become part of chromatographic operation and are complished via switching of valves. Preliminary sample handling can therefore be minimized. In most cases[1-4] the precolumns have been packed with silica-based bonded phase packings which limit the aqueous solvents that can be pumped through them to pH < 8.

Several methods have been developed for HPLC determination of lonazolac (LON), an antiinflammatory drug (Fig. 1)[5] in biological samples. These include cleanup procedures as following: liquid-liquid extraction[6], off-line precolumn enrichment[7] and on-line precolumn enrichment[8,9] with silica based reversed bonded packings.

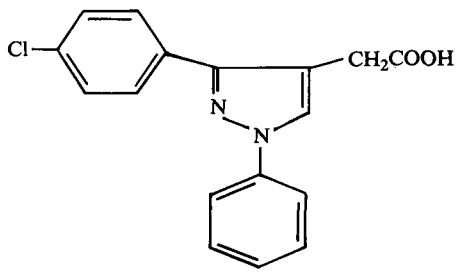

Fig. 1. Chemical structure of lonazolac (LON).

Amberlite XAD-2, which is compatible with all pH from 0 to 14, has been used in off-line[10,11] or on-line precolumns[12-14] for the determination of drugs in biological samples.

The present paper describes the use of XAD-2 precolumn in on-line sample treatment for the determination of trace levels of LON in human plasma.

EXPERIMENTAL METHODS

Reagents and standards

Acetonitrile (p.chr.), methanol (p.chr.), sodium dihydrogen phosphate (p.a.), disodium hydro-

gen phosphate (p.a.) and orthophosphoric acid (p.a.) were obtained from E. Merck (Darmstadt, F.R.G.). Water was distilled and then deionized with Nanopure II (Barnstead). LON Ca and N-phenylanthranilic acid (NPA) were obtained from Taepyoungyang Pharm. Co.(Suweon, Korea) and Tokyo Kassei Co.(Tokyl, Japan), respectively. The stock solutions were prepared in methanol (1 mg/m*l*) and stored at 4 °C. This stock solution was diluted with 0.05 M phosphate buffer (pH 2) as necessary and used to prepare the appropriate concentrations (2.0-200 μg/m*l* for LON). NPA(internal standard) was used as 15 μg/m*l* standard solution in methanol. Spiked plasma standards ranging 0.1-10 μg/m*l* of LON in plasma were then prepared in each assay by spiking 0.9 m*l* of plasma with 100 μl of LON working standards.

XAD-2 (20-50 mesh, Rohm & Hass) was microparticulated according to the method of Dieterle *et al.*[15] and the used particle size was 37-53 μm.

Instrumentation and chromatographic conditions

A schematic representation of column-switching system is shown in Fig. 2. The HPLC system consisted of a Spectra Physics Model SP 8800 pump (SantaClara, CA, U.S.A.), a Waters 501 pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne 7125 injector (Cotati, CA, U.S.A.), a Rheodyne 7000 switching valve and a Spectra Physics 8450 UV/VIS detector. Chromatogram recording and peak integrations were obtained through a Spectra Physics SP4290 integrator.

Precolumn was a 2 × 0.4 cm *i.d.* stainless steel column (Waters Assoc.) dry-packed with micronized XAD-2 (37-53 μm). The analytical column was a 25 × 0.45 cm *i.d.* stainless steel column prepacked with 10 μm Lichrosorb RP-18 (Spectra Physics).

Washing solvent was phosphate buffer (0.05 M, pH 2.0) and the flowrate was 1.0 m*l*/min. Mobile phase was 30% acetonitrile in phosphate buffer (0.05 M, pH 7) and the flowrate was 1.0 m*l*/min. The temperature was ambient and the wavelength of the UV detector was 280 nm.

Column-switching procedure for sample handling

Plasma (500 μl) was mixed with 50 μl of internal standard solution and 450 μl of 0.1 M phosphoric acid. After centrifugation (1 min at 1500 g), 200 μl of the diluted plasma sample was injected into precolumn and precolumn was flushed at flowrate of 1.0 m*l*/min for 8 min with washing solvent. The drugs were adsorbed on the precolumn and other components in plasma were washed out to waste.

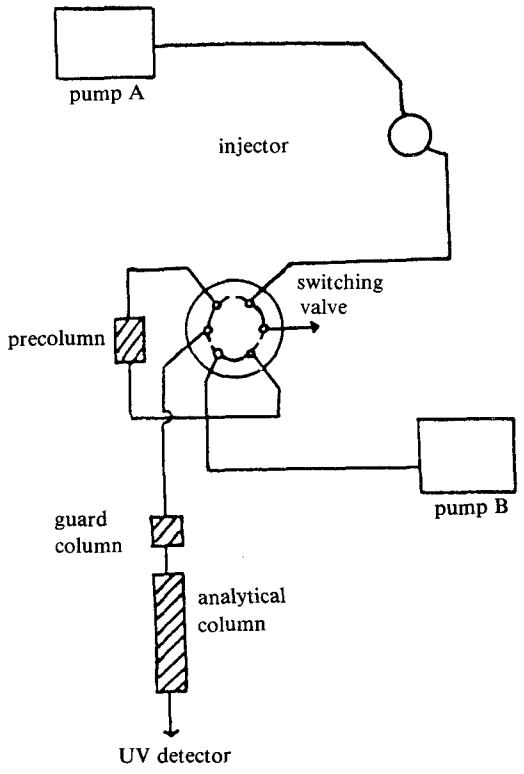

Fig. 2. Schematic diagram of a column-switching system.

Meanwhile, guard column and analytical column were equilibrated with mobile phase. The switching valve was turned to the mobile phase at 9 min after injection of plasma sample. The drugs enriched on the precolumn were eluted into analytical column by back-flush mode and separated efficiently. The valve was resetted to initial position after 4 min when LON and NPA were eluted completely into the analytical column.

RESULTS AND DISCUSSION

Sample pretreatment

In the first papers describing column-switching method[1,2,8,9], the plasma sample was injected without dilution or addition of an internal standard, using water or buffer as washing solvent. Subsequently, the usefulness of an internal standard and of dilution of the plasma to circumvent premature column blockage has been demonstrated several times.[3,4,16]

For very strongly protein-bound drugs the protein binding had to be decreased in the sample solution to obtain complete recoveries.[16,17]

Table I. Effect of sample dilution and addition of phosphoric acid on the enrichment of LON from plasma samples

Sample	Volume injected (μl)	Recovery (%)
Plasma	100	66.2
Plasma diluted (1:1) with 0.1M phosphoric acid	200	96.4
Plasma diluted (1:1) with 0.1M phosphate buffer (pH 7)	200	80.1

Dilution of the sample clearly increased the recovery of LON from 66.2% for non-diluted sample to 80.1% at 2-fold dilution of the sample with 0.1 M phosphate buffer (pH 7.0) (Table I). When phosphoric acid is added to the plasma sample, the protolysis of LON and the charge distribution on the protein molecules change, resulting in elimination of the protein binding ability. Consequently, the addition of phosphoric acid to plasma sample gave recovery of 96.4%.

Column-switching procedure

It is necessary that precolumn packing and washing solvent be chosen in such a way that LON is completely retained while the interfering components of plasma are un-retained on the precolumn.

XAD-2 is suitable as a precolumn packing because of its inertness at all pH and strong sorbent properties.

Fig. 3 shows the retention variation of LON versus methanol content in the mobile phase on XAD-2 (37-53 μm) for various pH. The variation curves log k' = f(x) [x is methanol percentage in mobile phase] are linear [18]. By extrapolation of these curves for x = 0, the capacity factor k' in pure aqueous buffer can be obtained.

The capacity factor k' of LON increases with decreasing pH because LON is an acidic drug (pKa = 4.3).[19] At pH 2.0, LON is present as nonionic form and the charges of amino acid groups on albumin change. Consequently, the majority of plasma components is relatively unretained on the precolumn in phosphate buffer (0.05 M, pH 2.0) and LON exhibits strong retention on XAD-2 ($k' > 10^6$). Therefore, phosphate buffer (0.05 M, pH 2.0) was used as washing solvent in the system.

Precolumn washing after plasma injection is important because endogenous interferences in plasma can be eliminated. Using a flowrate 1.0 ml/

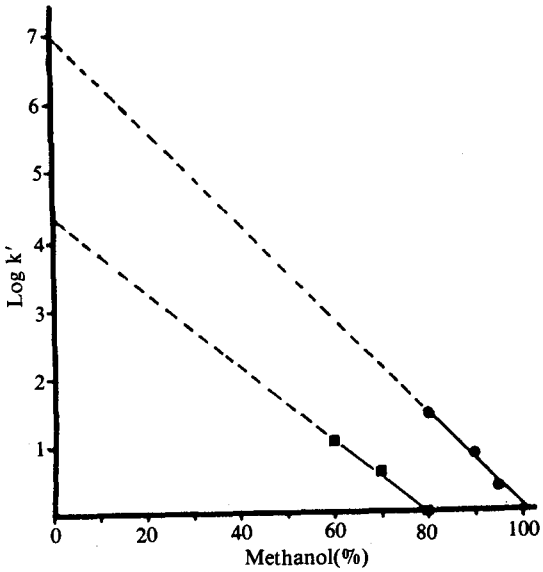

Fig. 3. Capacity factor (log k') of lonazolac as a function of mobile phase composition using methanol-water as the mobile phase.
stationary phase: XAD-2 (37-53 μm), column: 10 × 0.4 cm *i.d.* flowrate: 1.0 ml/min, detection: UV 280 nm. ● – ● pH 2.0 ■ – ■ pH 7.0

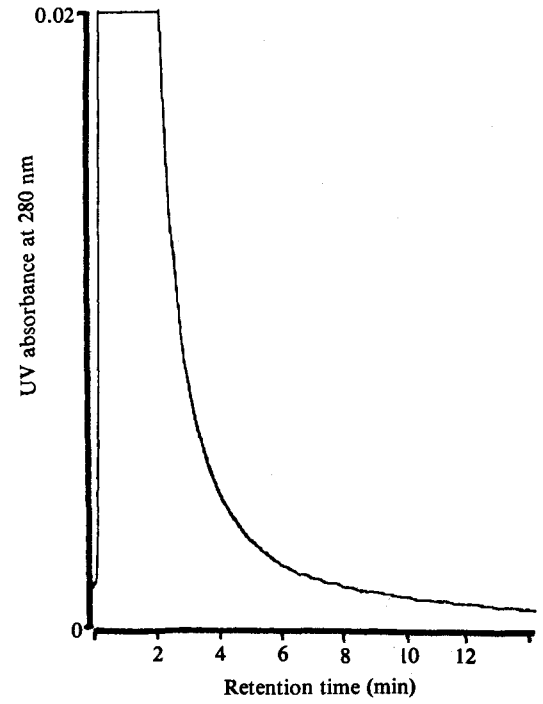

Fig. 4. Elution of plasma from XAD-2 as a function of washing solvent volume through the precolumn.

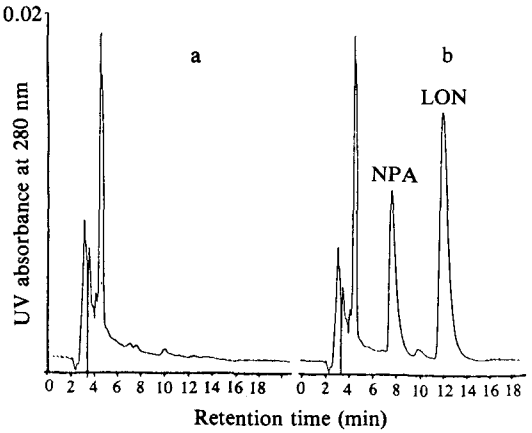

Fig. 5. Chromatograms of (a) a pooled human drug-free plasma by XAD-2 and (b) drug-free human plasma spiked with internal standard and 5 μg/ml LON.

min, the clean-up process was completed in less than 9 min. (Fig. 4). The effectiveness of washing solvent in removing the plasma components from the precolumn was shown in Fig. 5a.

Chromatography of LON

The choice of an octadecyl-silica packing for the analytical column was based on the high efficiency, ready availability and popularity of such bonded phase packings.

Optimization of the separation leads to use of the following mixture as the mobile phase: 30% acetonitrile in phosphate buffer (0.05 M, pH 7.0) (Rs = 4.32). (Fig. 5b) At pH 7.0, LON is present as an ionized form, and thus, is very soluble in the aqueous-organic mobile phase. This ensures their quantitative elution from the precolumn.

LON was retained on the top of precolumn due to its high capacity factor value. (k' at pH 2.0 $>10^6$) Adsorbed LON was eluted by backflush mode. Therefore, the influence of precolumn void volume on efficiency was negligible and the elution volume of LON and NPA was small.

The precolumn was replaced every 100 injections. The guard column was changed after 250-300 injections. Analytical column showed no decrease in efficiency after 300 injections.

Recovery test

In order to determine the recovery, two calibration curves based on the external standard method were plotted. One calibration curve was based on spiked plasma samples in the concentration range 0.1-10.0 μg/ml and the other was based on aqueous standards in the same range.

Table II. Recovery of LON from spiked plasma

Concentration (μg/ml)	LON peak heights (n = 4)		Recovery (%)
	Set A: aqueous standard	Set B: standard extracted from plasma	
0.1	38691	36992	95.6
0.5	183624	177155	96.5
1.0	371396	360709	97.1
2.0	781463	748997	95.8
5.0	1886944	1818876	96.4
10.0	3913622	3759893	96.1

Mean recovery: 96.3%

Regression line for set A: $y = 380581 \times -14843$, $r = 0.9998$

Regression line for set B: $y = 375291 \times -12967$, $r = 0.9999$

Mean recovery: $(375291/380581) \times 100 = 98.6\%$

Mean recovery determined by the two methods: 97.5%

The overall recovery was calculated by the two methods: (i) comparison of the slopes of the regression lines for the two sets and (ii) direct comparison of the peak areas (Table II).

As shown in Table II, mean recovery from peak area was 96.3%, recovery from regression line was 98.6% and overall mean recovery from the two methods was 97.5%.

linearity and limit of detection

Evaluation of the assay was carried out using six point calibration standards in the concentration range 0.1-10 μg/ml LON in plasma. The calibration plot of peak area ratios of LON/NPA versus the concentration of LON in plasma was linear with a correlation coefficient of 0.9991.

Under the described conditions, the limit of detection was 0.1 μg/ml LON in plasma injecting 0.2 ml of diluted plasma samples (100 μl plasma equivalents). Detection limit was taken as the amount of compound showing a signal-to-noise ratio greater than 3:1.

Reproducibility

The precision (defined as coefficient of variation, C.V.) and the accuracy (defined as the deviation between found and added concentration) of the method for LON were evaluated over the concentration range 0.1-10 μg/ml plasma. The intra-assay reproducibility was determined by analysing

Table III. Intra-assay reproducibility (n=4)

Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)	Deviation (%)
0.1	0.11	7.9	+6
0.5	0.49	4.2	−2
1.0	1.03	1.7	+3
2.0	2.05	1.7	+2.5
5.0	4.80	1.9	−4
10.0	10.0	4.0	0

Table IV. Inter-assay reproducibility (n=5)

Concentration added (μg/ml)	Concentration found (μg/ml)	C.V. (%)	Deviation (%)
0.1	0.11	8.2	+6
0.5	0.53	7.5	+6
1.0	1.03	4.7	+3
2.0	1.93	4.7	−3.5
5.0	4.79	2.8	−4.2
10.0	9.83	1.0	−1.7

four specimens of spiked plasma samples on the same day. The interassay reproducibility was obtained by analysing one specimen of a spiked plasma sample on five days over a period of three weeks.

The results are given in Table III and IV. C.V. were better than 4.0% at 0.5-10 μg/ml. However, slightly greater variation was found at 0.1 μg/ml.

interference study

Other drugs were chromatographed under the described condition.

The following drugs yielded no chromatographic peak near that of LON: acetoaminophen, alclofenac, diazepam, diclofenac, mefenamic acid, piroxicam, phenylbutazone, caffeine.

CONCLUSION

Amberlite XAD-2 was an ideal adsorbent for on-line trace enrichment of LON from microvolumes of biological samples without laborious sample manipulations. Column-switching HPLC method using micronized XAD-2 precolumn was developed for the determination of LON from plasma. This method was more rapid, sensitive and reproducible than the previous methods.

LITERATURE CITED

1. Voelter, W., Kronbach, T., Zech, K. and Huber, R.: A simple high performance liquid chromatographic precolumn technique for investigation of drug metabolism in biological fluids. *J. Chromatogr.* **239**, 475 (1982).
2. Little, C.J., Tompkins, D.J., Stahel, O., Frei, R.W. and Goewie, C.E.W.: Application of a microprocessor-controled valve-switching unit for automated sample cleanup and trace enrichment in high performance liquid chromatography. *J. Chromatogr.* **264**, 183 (1983).
3. Powell, W.S.: Precolumn extraction and reversed-phase high pressure liquid chromatography of prostaglandins and leukotrienes. *Anal. Biochem.* **164**, 117 (1987).
4. Wyss, R. and Bucheli, F.: Quantitative analysis of retinoids in biological fluids by high performance liquid chromatography using column switching I. Determination of isotretinoin, tretinoin and 4-oxo metabolites in plasma. *J. Chromatogr.* **424**, 303 (1988).
5. Riedel, V.R.: Pharmacologische Eigenschaften von Lonazolac-Ca, einer neuen Antiphlogistisch und antirheumatisch wirksamen substanz. *Arzneim. Forsch./Drug Res.* **31**(I), 655 (1982).
6. Battista, H.J., Wehinger, G. and Henn, R.: Separation and Identification of nonsteroidal antirheumatic drugs containing a free carboxyl function using high performance liquid chromatography. *J. Chromatogr.* **345**, 77 (1985).
7. Zech, K., Arnold, P., Liechti, H. and Ludwig, G.: HPLC in pharmacokinetics and metabolism studies. *Chromatographia* **12**(6), 354 (1979).
8. Huber, R., Zech, K., Woerz, M., Kronbach, T. and Voelter, W.: Direct injection of biological fluids; Application of HPLC analysis with precolumn enrichment to pharmacokinetic studies of lonazolac. *Chromatographia* **16**, 233 (1982).
9. Arnold, P.J., Guserle, R., Woerz, M. and Luckow, V.: Column-switching in HPLC. Determination of drug substances in biological material. *Chromatographia* **22**, 431 (1986).
10. Tjaden, U.R., Langenberg, J.P., Ensing, K., Bennekom, W.P.V., De Bruijn, E.A. and Van Oosterom, A.T.: Determination of mitomycin C in plasma, serum and urine by high performance liquid chromatography with ultraviolet

and electrochemical detection. *J. Chromatogr.* **232,** 355 (1982).

11. De Raat, W.K. and Van Ardenne, R.A.M.: Separation of organic compounds from urine in mutagenicity testing: Choice of sorbent. *J. Chromatogr.* **310,** 41 (1984).
12. Koch, D.D. and Kissinger, P.T.: Determination of serotonin in serum and plasma by liquid chromatography with precolumn sample enrichment and electrochemical detection. *Anal. Chem.* **52**(1), 113 (1980).
13. Hux, R.A., Mohammed, H.Y. and Cantwell, F.F.: Precolumns of Amberlite XAD-2 for direct injection liquid chromatographic determination of methaqualone in blood plasma. *Anal. Chem.* **54**(1), 113 (1982).
14 Niessen, W.M.A., Bergers, P.J.M., Tjaden, U.R. and Van der Greef, J.: Phase system switching as an on-line sample pretreatment in the bioanalysis of mitomycin C using supercritical fluid chromatography. *J. Chromatogr.* **454,** 243 (1988).
15. Dieterle, W., Faigle, J.W. and Mory, H.: Preparative reversed-phase liquid chromatography of polar and nonpolar metabolites on columns packed with micronized XAD-2 resin. *J. Chromatogr.* **168,** 27 (1979).
16. Arvidsson, T.: Effects of drug-protein binding on trace enrichment of drugs in blood plasma on short precolumns. *J. Chromatogr.* **439,** 353 (1988).
17. Wahlund, K.G. and Arvidsson, T.: Elimination of peak deformation in the liquid chromatographic separation of a strongly protein-bound drug from directly injected blood plasma samples. *J. Chromatogr.* **282,** 527 (1983).
18. Hennion, M.C., Picard, C., Combellas, C., Caude, M. and Rosset, R.: Simple relationships concerning mobile and stationary phases in normal- and reversed-phase chromatography. *J. Chromatogr.* **210,** 211 (1981).
19. Rainer, V.G., Kruger, U. and Klemm, K.: Synthese und Physikalisch-Chemische Eigenschaften von Lonazolac-Ca, einem neuen Antiphlogistikum/Antirheumatikum. *Arzneim. Forsch./Drug Res.* **31**(I), 649 (1981).