Journal of Chromatography, 575 (1992) 93–99
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6210

Automated high-performance liquid chromatography of iopentol in human plasma and whole blood using on-line dialysis as sample preparation

Alf T. Andresen*

Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo 3 (Norway)

Petter B. Jacobsen

Nycomed AS, P.O. Box 4220 Torshov, N-0401 Oslo 4 (Norway)

Knut E. Rasmussen

Institute of Pharmacy, University of Oslo, P.O. Box 1068, Blindern, N-0316 Oslo 3 (Norway)

(First received August 19th, 1991; revised manuscript received November 4th, 1991)

ABSTRACT

A fully automated high-performance liquid chromatographic method has been developed for the analysis of the radiographic contrast agent iopentol in human plasma and whole blood. This method is based on on-line sample preparation with dialysis followed by pre-column enrichment of the dialysate. The method was compared with a manual sample preparation method based on protein precipitation with tetrahydrofuran. The inter-assay and intra-assay variations and the limits of quantitation were the same for both methods. The on-line combination of dialysis and column-switching high-performance liquid chromatography was shown to be a reliable and time-saving technique for monitoring iopentol in human whole blood and plasma.

INTRODUCTION

Iopentol (Imagopaque), N,N′-bis(2,3-dihydroxypropyl)-5-[N-(2-hydroxy-3-methoxypropyl)acetamido]-2,4,6-triiodoisophthalamide, is a new non-ionic X-ray radiographic contrast agent. It is a chemical and functional analogue of iohexol (Omnipaque). Iopentol exhibits *exo–endo* isomerism, as shown in Fig. 1. Both iopentol and iohexol are highly soluble in water. They cannot be extracted from body fluids with organic solvents, and the high polarity of the compounds complicates solid-phase extraction from aqueous matrixes. High-performance liquid chromatography (HPLC) of iopentol and iohexol by reversed-phase systems is performed with a mobile phase containing a low concentration of organic modifier [1,2]. Sample preparation with protein precipitation and subsequent HPLC analysis have

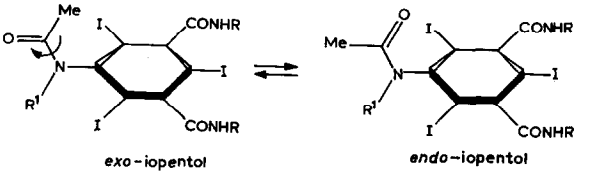

Fig. 1. *Exo-endo* isomerism in iopentol. R = $CH_2CHOHCH_2OH$; R^1 = $CH_2CHOHCH_2OMe$.

0378-4347/92/$05.00

been used for the analysis of these drugs in plasma. Normal doses of iopentol administered for radiographic examination are in the range 0.4–2 g/kg body weight [3], and plasma concentrations monitored in pharmacokinetic studies are in the range 0.5–1000 μg/ml.

When monitoring iopentol in blood a method for the direct determination in whole blood could be favourable, particularly for pharmacokinetic studies in small animals (*e.g.* mice) containing small amounts of blood. No such method has been availabe. There has also been a need for a fully automated method able to analyse iopentol in plasma and whole blood from humans participating in clinical trials.

By using dialysis as a purification step prior to HPLC, whole blood and plasma analysis is considerably simplified, and fully automated methods can be worked out [4–8]. Crude samples are injected into a dialyser. Only low-molecular-mass compounds diffuse through the dialysis membrane, and the analytes collected in the dialysate are concentrated on a trace-enrichment column. The trace-enrichment column is connected to the HPLC system in a column-switching system. This is the principle of the fully automated ASTED (automated sequential trace enrichment of dialysates) system. Schematic representations of this system have been shown in several publications [4,6].

This paper reports a fully automated method for the analysis of iopentol in human plasma and whole blood based on the ASTED system for sample preparation. The performance of the ASTED method is compared with a manual protein precipitation method.

EXPERIMENTAL

Chemicals

Iopentol was supplied by Nycomed (Oslo, Norway). HPLC-grade acetonitrile, methanol and tetrahydrofuran were supplied by Rathburn (Walkerburn, UK). Triton X-100 was obtained from Merck (Darmstadt, Germany). HPLC-grade water was obtained from a Milli-Q (Millipore, MA, USA) water purification system.

Preparation of standards and samples

Citrated blood from healthy donors was obtained from The Red Cross Blood Centre (Oslo, Norway). Plasma was prepared by centrifuging the blood for 15 min at 1920 *g*. A 10 mg/ml working solution of iopentol was prepared in plasma. Spiked plasma and whole blood standards (0.5–1000 μg/ml) were prepared from the working solution.

ASTED method

The sample preparation system was a Gilson ASTED unit (Gilson Medical Electronics, Villiers-le-Bel, France) consisting of a Model 231 autosampling injector, two 401 diluters equipped with 1-ml syringes and one flat-bed dialyser with a donor channel volume of 100 μl and a recipient channel volume of 175 μl, fitted with a Cuprophan membrane, 15 000 dalton molecular mass cut-off. An automated six-port Model 7010 valve (Rheodyne, Berkeley, CA, USA) connected a trace-enrichment column (or a 50-μl sample loop) either with the recipient channel of the dialyser or with the mobile phase used in the analytical column of the HPLC system. The trace-enrichment column (5.0 mm × 3.0 mm I.D.) from Polymer Labs. was packed with 5 μm particle size polystyrene–divinylbenzene.

Protein precipitation method

A 500-μl aliquot of plasma was added to 250 μl of tetrahydrofuran and was vigorously shaken. After 20 min the sample was centrifuged for 15 min at 12 000 *g*. The supernatants were diluted five-fold with water prior to HPLC analysis. All steps were performed at 4°C.

High-performance liquid chromatography

The ASTED was coupled to an LC 6A (Shimadzu, Kyoto, Japan) HPLC pump. The UV detector was a Model SPD-6A (Shimadzu) with an 8-μl flow cell operated at 244 nm. Peak heights were recorded on a Chromatopac C-R3A integrator (Shimadzu). The analytical column (250 mm × 4.6 mm I.D.) was packed with 5 μm particle diameter RP-18 Spheri-5 (Brownlee Columns, Applied Biosystems). The mobile phase was

tetrahydrofuran (THF)–water (2.5:97.5, v/v) delivered at a flow-rate of 1.0 ml/min.

Analyses of the protein-precipitated samples were performed with a Spectra Physics SP 8800-020 pump (San Jose, CA, USA) and an SP 8540-010 UV–VIS detector (Spectra Physics) with a 4.5-μl flow cell operated at 244 nm. The automatic sample processor was a Gilson 232-401 with a Model 7010 valve (Rheodyne) with a 10-μl sample loop. The analytical RP-18 column and the mobile phase were the same as described above. Data handling (integrations and calculations) was performed by a PE Nelson Access*Chrom gas chromatography–liquid chromatography data system (Perkin Elmer Nelson, Cupertino, CA, USA).

ASTED procedure

Sample loading. One diluter was used to inject 100 μl of plasma or whole blood into the donor channel of the dialyser, with the six-port valve in the load position.

Dialysis and trace enrichment. The sample was held static in the donor channel for 7 min 10 s while the other diluter transported 1.5 ml of distilled water in the pulsed mode through the recipient channel of the dialyser and into the pre-column, with the injection valve still in the load position. The recipient solution was transported through the dialyser at an average flow-rate of 0.3 ml/min and was divided into 8.6 pulses of 175 μl (the volume of the recipient channel of the dialyser). After each pulse the recipient solution was held static in the recipient channel for 13 s.

Injection and purging. Upon switching of the six-port valve to the inject position the analytes on the trace-enrichment column were brought into the analytical column by elution for 4 min with the HPLC mobile phase. The recipient side of the dialyser was then washed with 2 ml of water. The donor side of the dialyser was simultaneously washed with 2 ml of water containing 100 mg/l Triton X-100.

Regeneration. After 4 min the six-port valve was switched back to the load position to bring the pre-column back to the recipient channel of the dialyser. After regeneration of the trace-enrichment column with 1 ml of water, the next sample was injected into the dialyser. Automated injections were performed every 20 min.

Validation of the procedures

The standard curves, accuracy and precision of the method were evaluated by analysis of spiked plasma and whole blood samples. The calibration graphs were based on external calibration standards measuring the peak heights *versus* concentrations in plasma or whole blood.

RESULTS AND DISCUSSION

Dialysis and trace enrichment

In a fixed dialysis cell, dialysis efficiency is influenced by the dialysis time and the concentra-

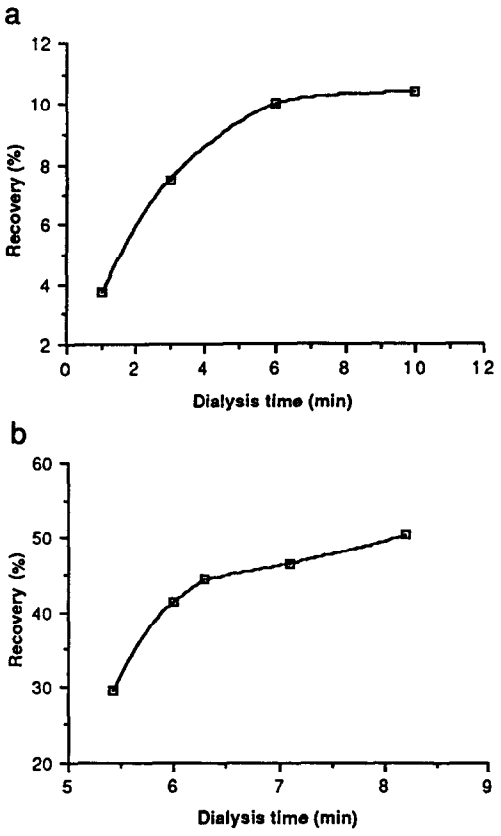

Fig. 2 (a) Recovery of iopentol from plasma after static–static dialysis. Water was used as recipient. (b) Recovery of iopentol from plasma after static-pulsed dialysis. A constant volume of 1.5 ml of water was aspirated in pulses through the recipient channel of the dialyser.

tion gradient across the membrane. It is known that low dialysis efficiencies are obtained during static–static dialysis [9]. By maintaining a steep concentration gradient across the membrane, such as in static-pulsed dialysis or static-continuous dialysis, high dialysis efficiencies are obtained in a short time [10–13]. In these modes the dialysate is concentrated on a trace-enrichment column before injection into the HPLC column. Iopentol is difficult to enrich on a solid-phase extraction column. However, the concentration range to be monitored in plasma is high, and static–static dialysis was therefore investigated as sample preparation method. After dialysis the dialysate was transported in one single pulse to overfill a 50-μl sample loop and injected into the HPLC column. As shown in Fig. 2a a recovery of 10% was obtained from plasma during 10 min of dialysis. The chromatograms of drug-free plasma and plasma spiked with 10 μg/ml iopentol are shown in Fig. 3. The broad fronts in the chromatograms and the low sensitivity made this method unsuitable.

To increase dialysis efficiency static-pulsed dialysis with concentration of the dialysate on a trace–enrichment column was investigated. No silica-based material was found which was able to retain iopentol. Fig. 4 shows the breakthrough

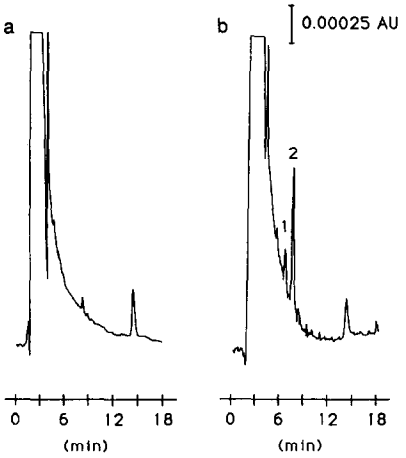

Fig. 3. Chromatograms of (a) drug-free plasma and (b) plasma spiked with 10 μg/ml iopentol after static–static dialysis and loop injection. Detection: UV at 244 nm. Peaks: 1 = *endo*-iopentol; 2 = *exo*-iopentol. For chromatographic conditions, see text.

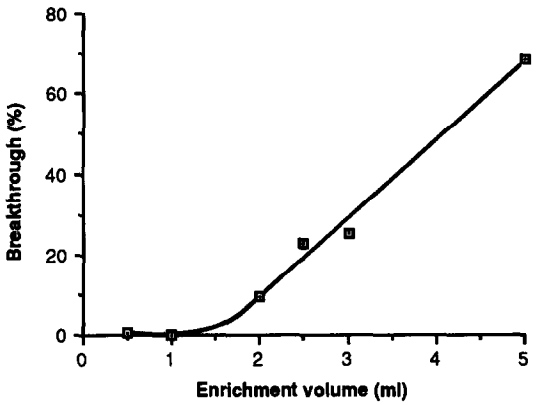

Fig. 4. Breakthrough of iopentol from the trace-enrichment column after elution of the column with water.

of iopentol from a trace-enrichment column packed with polystyrene after elution of the column with water. In this study 10 μg of iopentol was directly injected on to the trace-enrichment column, which was twice the amount that was enriched during analysis of real samples. A breakthrough volume of 1.5 ml was found. A dialysis procedure then had to be developed which would give a satisfactory dialysis efficiency with 1.5 ml of water as recipient. Fig. 2b shows the recovery of iopentol from plasma when the ASTED was operated in the static-pulsed mode. The sample was held static in the donor channel of the dialyser while a constant volume of 1.5 ml of water was delivered in pulses of 175 μl through the recipient channel. The recovery was about 50% when the sample was dialysed for 7 min 10 s. Only a slight increase in dialysis efficiency was observed when the samples were dialysed longer. The dialysis efficiencies were determined by peakheigt measurements from a calibration graph setup after direct injection of iopentol into the analytical column. The same dialysis efficiencies were obtained after dialysis of standards dissolved in water as of standards dissolved in plasma. Iopentol is not bound to proteins, and this result shows that the matrix does not influence the dialysis efficiency of a non-protein-bound drug in plasma. Dialysis of whole blood showed a slightly lower dialysis efficiency (47%), probably because the presence of blood cells in the sample reduces diffusion.

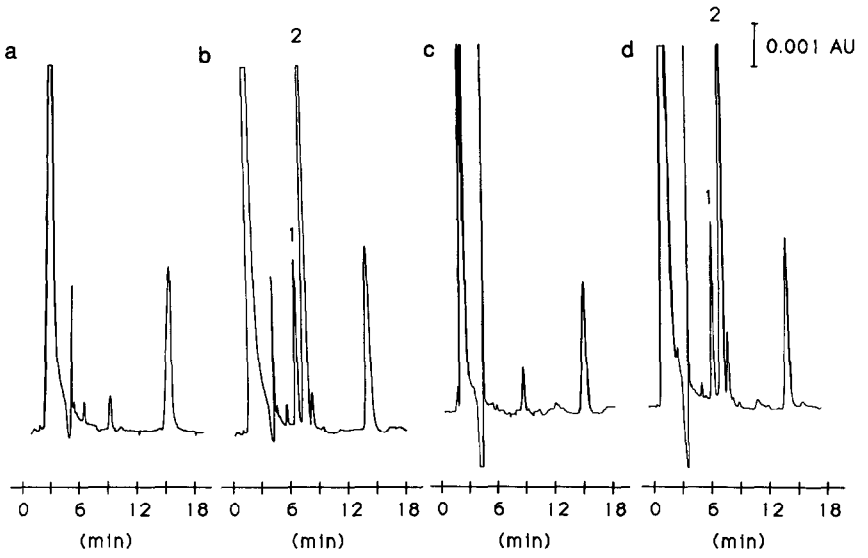

Fig. 5. Chromatograms of (a) drug-free plasma, (b) plasma spiked with 10 μg/ml iopentol, (c) drug-free whole blood and (d) whole blood spiked with 10 μg/ml iopentol after static-pulsed dialysis and trace enrichment of the dialysate on the pre-column. Detection: UV at 244 nm. Peaks: 1 = *endo*-iopentol; 2 = *exo*-iopentol. For chromatographic conditions, see text.

HPLC separation

The HPLC system should separate the *exo* and *endo* isomers of iopentol in a reasonably short time and elute the compounds free from interferences from endogenous substances in plasma and whole blood. A satisfactory separation was achieved with an RP-18 column and a mobile phase consisting of 2.5% THF in water. Fig. 5 shows chromatograms of plasma and whole blood spiked with 10 μg/ml iopentol. Reversed-phase chromatography of plasma or whole blood samples with a mobile phase containing a low concentration of organic modifier, is likely to show the presence of endogenous substances. Drug-free plasma and whole blood (Fig. 5) also showed the presence of three endogenous substances eluted at approximately 6, 9 and 15 min. However, these substances did not interfere with the analysis of iopentol. On-line dialysis and concentration of dialysate as sample preparation give sufficient clean-up of the samples with narrow fronts in the chromatograms and no significant difference between chromatograms from whole blood and plasma.

Protein precipitation

Plasma proteins were precipitated with THF. Precipitation by acid (10% trifluoroacetic acid) was also tested, but this agent gave a broader front and more interfering endogenous compounds in the chromatogram. Owing to the high final concentration of THF in the sample (33.3%) a five-fold dilution of the sample with water prior to injection into the HPLC system was necessary to give good chromatograms.

Fig. 6 shows the chromatograms of drug-free

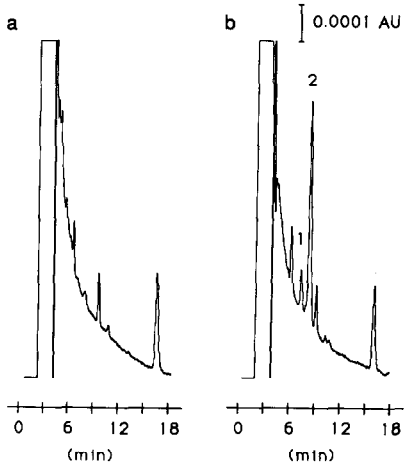

Fig. 6. Chromatograms of (a) drug-free plasma and (b) plasma spiked with 10 μg/ml iopentol after protein precipitation with THF. Detection: UV at 244 nm. Peaks: 1 = *endo*-iopentol; 2 = *exo*-iopentol. For chromatographic conditions, see text.

TABLE I

INTRA-ASSAY VARIATIONS AFTER ASTED AND PROTEIN PRECIPITATION (THF) SAMPLE TREATMENT, EXPRESSED AS MEAN OF PARALLEL SAMPLES ± SAMPLE STANDARD DEVIATION (S.D.) AND PERCENTAGE RELATIVE STANDARD DEVIATION (R.S.D.)

Method	Matrix	Calculated concentration (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
ASTED	Whole blood	0.50	0.573 ± 0.099	2.8
(n = 5)[a]		1.00	1.17 ± 0.072	6.1
		10.0	9.92 ± 0.32	3.2
		500	476 ± 3.5	0.7
ASTED	Plasma	0.50	0.634 ± 0.080	16.0
(n = 5)[a]		1.00	1.10 ± 0.012	1.2
		10.0	10.7 ± 0.15	1.4
		500	508 ± 4.2	0.8
THF	Plasma	0.50	0.621 ± 0.099	16.0
(n = 10)[a]		1.00	1.04 ± 0.09	9.1
		10.0	10.9 ± 0.4	4.1
		500	496 ± 8.1	1.6

[a] Number of parallel sample measurements.

plasma and a plasma spiked with 10 μg/ml iopentol. Endogenous compounds similar to those present after on-line dialysis (Fig. 5) can be seen. No interferences with the *exo-* and *endo*-iopentol peaks were observed. The *exo- and endo*-iopentol peaks were, however, eluted on the tail of the solvent front.

Validation of the methods

The calibration graphs for both methods were based on peak-height measurements of external standards. Several analogues of iopentol, iohexol and other polar substances were tested as possible internal standards, but no suitable internal standard was found. Linear calibration graphs

TABLE II

INTER-ASSAY VARIATIONS AFTER ASTED AND PROTEIN PRECIPITATION (THF) SAMPLE TREATMENT, EXPRESSED AS MEAN OF PARALLEL SAMPLES ± SAMPLE STANDARD DEVIATION (S.D.) AND PERCENTAGE RELATIVE STANDARD DEVIATION (R.S.D.)

Method	Matrix	Calculated concentration (μg/ml)	Measured concentration (mean ± S.D.) (μg/ml)	R.S.D. (%)
ASTED	Whole blood	1.00	1.15 ± 0.27	24.0
(n = 6)[a]		10.0	10.0 ± 0.56	2.7
		500	487 ± 3.5	0.7
ASTED	Plasma	0.50	0.595 ± 0.099	17.0
(n = 6)[a]		1.00	1.06 ± 0.025	2.3
		10.0	10.5 ± 0.18	1.7
		500	494 ± 14	2.8
THF	Plasma	0.50	0.618 ± 0.204	33.0
(n = 3)[a]		1.00	1.04 ± 0.05	4.5
		10.0	10.8 ± 0.03	0.3
		500	502 ± 11	2.2

[a] Number of parallel sample measurements.

were obtained in the concentration range 0.5–100 μg/ml iopentol. The correlation coefficients were 0.998 or better. Whole blood or plasma samples with concentrations above 100 μg/ml were diluted with saline solution before injection. Tables I and II show the intra-assay and inter-assay variations of the ASTED and the protein precipitation method. The limits of quantitation (LOQ) for the determination of iopentol in plasma and whole blood were set to the concentration giving 20% relative standard deviation (R.S.D.). These limits were estimated by curve-fitting to the inter- and intra-assay R.S.D. data by the PC program GraphPAD INPLOT. All the measurement series resulted in LOQ values of about 0.5 μg/ml.

Comparison of the ASTED method and the protein precipitation method

Compared with the protein precipitation method, the on-line dialysis method is superior concerning time of work and analysis. The sample clean-up procedure is performed automatically within the analytical run time. Direct analysis on whole blood is an obvious advantage. The inter-assay and the intra-assay variations and the LOQ are about the same for both methods. The column lifetime is very good for the ASTED method. No chromatographic changes could be seen after more than 1000 injections without changing the trace-enrichment column or the dialysis membrane. After 250 injection of the protein–precipitated samples the solvent front peak became broader and the column had to be cleaned.

CONCLUSION

The results presented in this paper show that HPLC analysis of iopentol in plasma or whole blood can be fully automated in an on-line dialysis and column-switching system. The method is shown to be highly reliable. Protein precipitation also gives satisfactory results, but has the disadvantage of being very time-consuming. The ASTED method should be considered when a large number of biological samples are analysed.

REFERENCES

1 J. Edelson, G. Palace and G. Park, *J. Chromatogr.*, 274 (1983) 428.
2 K. Skinnemoen, R. Fagervoll and T. Jacobsen. *Acta Radiol.*, 370 (Suppl.), (1987) 23.
3 A. Waaler, N. P. Jørgensen, B. Koksvik, K. Skinnemoen and A. Andrew, *Acta Radiol.*, 370 (Suppl.), (1987) 113.
4 D. C. Turnell and J. D. H. Cooper, *J. Chromatogr.*, 395 (1987) 613.
5 A. J. J. Debets, W. Th. Kok, K. P. Hupe and U. A. Brinkman, *Chromatographia*, 30 (1990) 361.
6 J. D. H. Cooper, D. C. Turnell and B. Green, *J. Chromatogr.*, 456 (1988) 53.
7 T. Agasøster and K. E. Rasmussen, *J. Chromatogr.*, 564 (1991) 171.
8 A. D. Dale and S. E. Turner, *J. Pharm. Biomed. Anal.*, 8 (1990) 1055.
9 D. C. Turnell and J. H. Cooper, *J. Autom. Chem.*, 7 (1985) 177.
10 J. D. H. Cooper, D. C. Turnell, B. Green, D. J. Wright and E. J. Coombes, *Ann. Clin. Biochem.*, 25 (1988) 577.
11 M. M. L. Aerts, W. M. J. Beek and U. A. Th. Brinkman, *J. Chromatogr.*, 500 (1990) 453.
12 T. H. Hoang, A. T. Andresen, T. Agasøster and K. E. Rasmussen, *J. Chromatogr.*, 532 (1990) 363.
13 A. T. Andresen and K. E. Rasmussen, *J. Liq. Chromatogr.*, 13 (1990) 4067.