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Clean-up of plasma extracts by gel permeation chromatography during analysis of isosorbide nitrates by capillary gas chromatography

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ABSTRACT

This work describes how gel permeation chromatography (GPC) can be used for sample clean-up to reduce the fouling of the column in an automated on-column injector. The analytes were isolated from plasma together with the internal standard (isomannide dinitrate) by liquid–liquid extraction on Extrelut silica columns. The extracts were evaporated and reconstituted in tetrahydrofuran for separation of the analytes from non-volatile plasma components by GPC on a styrene–divinylbenzene column with 100 Å pore size. A programmable autosampler with an additional three-way valve was used for injection and fraction collection. The molecular weight fraction between 100 and 700 a.m.u. was collected and transferred to the on-column autosampler for capillary gas chromatography on a 30-m column butt-connected to a 0.2-m pre-column. The pre-column was replaced after 50 sample injections. When the GPC purification was excluded from the work-up procedure a deposit of non-volatile components was formed at the injection zone of the pre-column which resulted in excessive peak-tailing after only five or six injections of plasma extract. The limit of determination was 0.2 ng/ml plasma for isosorbide dinitrate and 0.4 ng/ml for the mononitrates.

INTRODUCTION

The vasodilator isosorbide dinitrate (ISDN) is rapidly metabolized in the liver and only a fraction of an oral dose reaches the systemic blood circulation as unchanged ISDN. Its primary metabolites isosorbide-5-mononitrate (ISMN-5) and isosorbide-2-mononitrate (ISMN-2) which are pharmacologically active are eliminated slower than the parent compound and circulate in the blood at higher concentrations [1]. The mononitrates are metabolized further to isosorbide and by glucuronidation to isosorbide-5-mononitrate-2-glucuronide [1]. Formation of small amounts of isomannide mononitrate and isoiodide mononitrate has also been reported [2]. For biopharmaceutical investigations it is necessary to quantify the analytes in plasma down to 1 ng/ml (ISDN) or a few ng/ml of the mononitrates.

ISDN has no intrinsic properties for sensitive detection by common detectors for liquid chromatography (LC). Detection limits of 2.5 ng for ISDN have been

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reported using post-column photolysis and electrochemical detection of the photolysis product nitrate [3]. This detection method has not been applied to biological samples and the sensitivity is not sufficient in the low ng/ml range. The thermal energy analyzer based on the chemiluminescence of nitrogen dioxide radicals produced by pyrolysis of the organic nitrates offers high sensitivity and an LC method for plasma samples has been described [4].

The most commonly used methods have been based on gas chromatography (GC) with electron-capture detection (ECD), first with packed columns [5,6] and later on capillary columns [2,7–9] without derivatization of the mononitrates. GC–tandem mass spectrometry (MS–MS) has also been used to determine isosorbide and isosorbide-5-mononitrate-2-glucuronide [10].

ISDN, chemically related to nitroclycerine and thermodynamically unstable, is sensitive to decomposition during GC and it is essential to have an inert and clean injector. When a non-selective extraction method such as liquid–liquid extraction is used, non-volatile sample components are co-extracted and deposited at the beginning of the column causing adsorption and resulting in non-linear calibration curves [7].

Sample purification by gel permeation chromatography (GPC) appears to be a logical method for elimination of non-volatile components with higher molecular weight than the analytes. One example is the GPC separation used on-line with GC for determination of polymer additives in polymers [11].

This work describes how GPC can be used in an automated manner for clean-up of plasma extracts prior to capillary GC of isosorbide nitrates.

EXPERIMENTAL

Chemicals and reagents

ISDN and ISMN-5 were obtained from Bofors Chemicals (Bofors, Sweden). ISMN-2 was obtained from Graesser Lab. (Sandycroof, Deeside, U.K.) and isomannide dinitrate was synthesized at the Department of Organic Chemistry at Kabi (Stockholm, Sweden). 2-Nitrobenzyl alcohol (NBA) was obtained from Fluka (Buchs, Switzerland). Dichloromethane and tetrahydrofuran, LiChrosolv grade, and disposable silica-gel columns, Extrelut-1, were obtained from Merck (Darmstadt, Germany). Dichloromethane was glass-distilled prior to use.

Instrumentation

Sample injection on the GPC column and fraction collection were performed in the same instrument by modification of a Gilson autosampler (Model 321, Gilson, Villiers-le-Bel, France). A three-way valve (Model LFYA; Lee Instac, Westbrook, CT, U.S.A.) was installed between the sampling needle and the syringe of the autosampler (dilutor/dispenser). The GPC column (300 mm × 7.8 I.D.) was packed with 10-μm Ultrastyragel with 100 Å pore size (Waters, Millipore, Milford, MA, U.S.A.). The eluent, tetrahydrofuran, was pumped at 1.5 ml/min with a Constametric pump (Model Bio 3000, LDC). The sample injections and fraction collections were all controlled by the programmable autosampler.

The gas chromatograph (Varian, Model 3500) was equipped with an on-column injector (Varian, Model 8035), an electron-capture detector and a GC-protector

(J & W Scientific, Folsom, CA, U.S.A.). A 0.2 m pre-column of the same type as the analytical column was butt-connected to a fused-silica column (30 m × 0.32 mm I.D.) coated with a 0.25-μm film thickness of the non-polar silicone phase DB-5 (J & W Scientific). Hydrogen was used as carrier gas at a flow-rate of 70 cm/s at 60°C and nitrogen was used as make-up gas (30 ml/min) to the detector, which was set to 250°C. The temperature programme of the injector and column is presented in Fig. 3. The peak areas were integrated by a Model 6000 laboratory data system from Nelson Analytical (Cupertino, CA, U.S.A.).

Sample preparation

Human blood samples were taken in heparinized Venoject tubes, cooled in an ice-bath and centrifuged within 30 min after sampling. The serum fraction was transferred to polypropylene tubes and the samples were stored at −20°C until analysis.

Plasma (0.9 ml) or blood diluted 1:2 with water was mixed with 0.1 ml internal standard solution (isomannide dinitrate, 50 ng/ml) and pipetted onto an Extrelut column. After 5 min, 6 ml dichloromethane were added to elute the analytes to a 10-ml glass-tube. The samples were put in an ice-bath and the solvent was evaporated with a stream of nitrogen. The residue in the tube was taken up in 0.2 ml terahydrofuran and transferred to micro-vials for the automatic injector. A 100-μl sample of the extract was injected onto the GPC column and the fraction between 6.9 and 8.4 ml was collected in another sample vial for the GC injector. The retention volume of the analytes was checked each day by injection of nitrobenzyl alcohol with UV detection at 254 nm. The retention volume of the analytes was 7.7 ml and the fraction between 6.9 and 8.4 ml was collected. The vial containing the collected fraction was manually transferred to the tray of the autosampler on the gas chromatograph, and 1 μl was injected for analysis.

Calibration was performed with spiked blank plasma purified along with unknown samples. The following levels were used for calibration; ISDN: 0.5, 1, 2.5, 3.5, 5.5, 9 and 10 ng/ml; ISMN-2: 2.5, 5, 10, 15, 25, 40 and 50 ng/ml; ISMN-5: 15, 25, 55, 80, 140, 225 and 275 ng/ml.

RESULTS AND DISCUSSION

Sample preparation

Liquid–liquid extraction on Extrelut columns was a convenient and rapid method for extraction of the analytes to dichloromethane from plasma. The solvent was evaporated on an ice-bath to avoid losses due to evaporation of the volatile analytes. The residue had to be taken up into tetrahydrofuran within 15 min after they had been blown to dryness to avoid losses due to evaporation of the analytes. The recovery of ISMN-2, ISMN-5 and ISDN in the extraction was checked in a separate experiment where the internal standard was added after the Extrelut extraction and evaporation steps, and the area ratios were compared with unextracted standards dissolved in tetrahydrofuran. Quantitative recovery was obtained for all of the analytes studied (see Table I). Calibration was performed by addition of the internal standard to spiked blank samples which resulted in linear calibration curves with good reproducibility between days (see Table II).

TABLE I

RECOVERY AND PRECISION

Compound	Concentration added (ng/ml)	Recovery (%)	n	Precision (R.S.D., %)	Detection limit (ng/ml)
ISDN	4.5	99.2	7	1.5	0.2
ISDN	50	101	8	2.8	
ISMN-2	4.5	98.9	7	2.7	0.4
ISMN-2	50	101	9	4.0	
ISMN-5	4.5	97.9	7	2.0	0.4
ISMN-5	50	103	9	2.5	

Stability of ISDN in blood and plasma

The stability of ISDN in fresh whole blood at 37°C was studied by adding ISDN and analyzing whole blood and plasma after different incubation times. The hematocrit of the blood was also measured to obtain the volume ratio of erythrocytes and plasma fractions. ISDN was rapidly distributed to blood cells within 1 min and then the concentration slowly decreased with approximately 25% reduction within 30 min (Fig. 1). The distribution coefficient between blood cells and plasma (C_c/C_p) was calculated to be 1.24 ± 0.02 (S.D.) from the blood concentrations, plasma concentrations and the hematocrit value according to the equation:

$$C_c/C_p = C_b\,(h \times C_p) - 1/h + 1$$

where C_c = ISDN concentration within the cell fraction, C_p = ISDN concentration in the plasma fraction, C_b = ISDN concentation in the whole blood and h is the cell fraction volume (hematocrit value %/100). The hematocrit value was 44% for the blood used in the experiment.

TABLE II

REPRODUCIBILITY OF CALIBRATION CURVES

Least-squares regression analysis of concentration (ng/ml) *versus* peak-area ratio of analyte and internal standard. A = Slope, B = intercept and r = correlation coefficient

Day	ISMN-2			ISMN-5			ISDN		
	A	B	r	A	B	r	A	B	r
1	0.713	0.008	0.9997	0.595	0.03	0.9997	0.901	−0.001	0.9999
2	0.704	0.02	0.9999	0.599	0.02	0.9996	0.905	−0.002	0.9999
3	0.715	0.005	0.9997	0.603	0.02	0.9999	0.923	0.000	0.9999
4	0.714	0.02	0.9999	0.592	0.02	0.9997	0.906	−0.002	0.9999
5	0.733	0.02	0.9991	0.614	0.01	0.9997	0.918	−0.002	0.9997
6	0.747	0.01	0.9998	0.613	0.03	1.0000	0.923	−0.002	0.9996
7	0.756	0.01	0.9975	0.612	0.01	0.9998	0.909	0.002	0.9993
8	0.689	0.01	0.9995	0.600	0.02	1.0000	0.907	0.000	1.0000
Mean	0.721	0.0129		0.604	0.02		0.912	−0.0008	
S.D.	0.0223	0.0061		0.0088	0.0076		0.0086	0.0014	

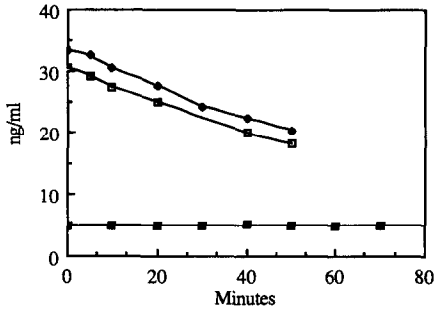


Fig. 1. Concentration of ISDN in blood (upper curve) and plasma (middle curve) after incubation at different times at 37°C, and the stability of ISDN in plasma at 22°C (lower line). Whole blood and plasma were spiked with ISDN to a concentration of 31 and 5.3 ng/ml, respectively.

A similar stability study was performed for ISDN in plasma at ambient temperature (22°C). ISDN was stable in plasma for at least 70 min (Fig. 1) ISDN is probably distributed to blood cells and hydrolyzed by esterases. Thus blood samples have to be cooled and centrifuged soon after sampling to obtain accurate results. Stability studies of frozen control samples showed that ISDN was stable at −20°C for at least 5 months.

GPC purification of plasma extracts

Initially we tried to inject the plasma extracts into the gas chromatograph after the Extrelut extraction and evaporation step but excessive tailing and adsorption problems occurred after only five or six injections. Tailing was especially pronounced

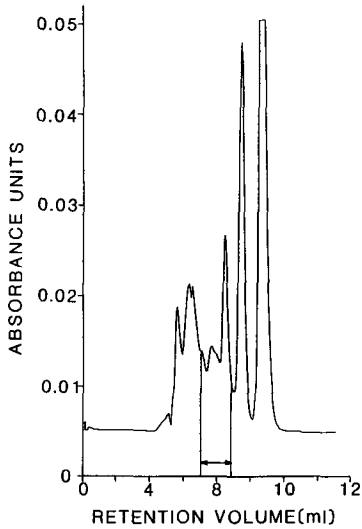


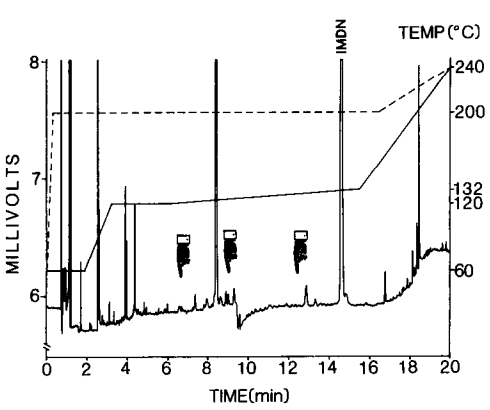
Fig. 2. UV chromatogram (254 nm) obtained after injection of 100 μl of plasma extract on the GPC column. The fraction between 6.9 and 8.4 ml was collected for analysis by GC.

for the mononitrates with a free hydroxy group. The problem with injector contamination has also been described by Ahnoff and Holm [7] and Lutz *et al.* [2] using splitless injection of plasma extracts. In general, cold on-column injection is supposed to be more gentle compared with splittless injection with flash evaporation of the sample, but the on-column injector was contaminated after fewer injections, probably due to a more concentrated zone of non-volatile material deposited at the beginning of the narrow column.

GPC purification of the extracts was then investigated for separation of the analytes from non-volatile plasma components such as lipids and other compounds with high molecular weight. The ultrastyragel column gave complete separation of toluene, ISDN and coenzyme Q_{10}. Coenzyme Q_{10} with a molecular weight of 862 was chosen as a representative of the lipoprotein fraction in plasma and was readily detected by UV for chromatographic registration. The internal standard isomannide dinitrate and the analytes co-eluted as one chromatographic peak, slightly broadened due to a minor difference in retention between the mono- and dinitrates. The elution fraction corresponding to a molecular weight of 100 to 700 a.m.u. (6.9–8.4 ml) gave quantitative collection of the analytes. A UV chromatogram of a plasma extract is presented in Fig. 2. Tetrahydrofuran was selected as the eluent for the GPC column to minimize hydrophobic interaction between sample components and the column packing material. This eluent was also a suitable solvent for the following step involving GC.

Capillary gas chromatography

Since it is known from previous work [7] that isosorbide dinitrates decompose at column temperatures above 150°C, hydrogen was chosen as the carrier gas to enable the use of higher flow-rates at a lower column temperature. The capillary column (DB-5) was of the same type as used by Lutz *et al.* [2], which is similar to the



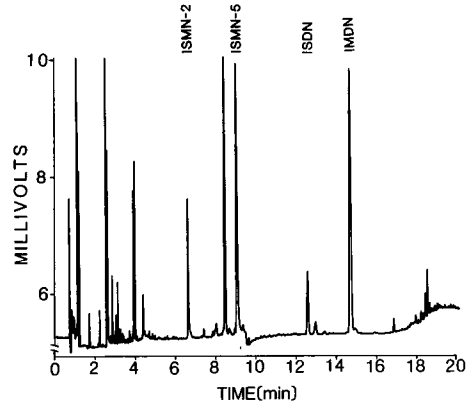


Fig. 3. GC profile obtained after injection of blank plasma sample. The injector temperature is shown by the broken line and the column temperature by the solid line. The hands show the retention time of the analytes in Fig. 4.

Fig. 4. GC profile obtained after injection of a patient plasma sample taken 2 h after a 10-mg oral dose of ISDN, with plasma of concentrations of 17.1, 70.6 and 8.5 ng/ml for ISMN-2, ISMN-5 and ISDN, respectively.

CPSil 5 column used by Ahnoff and Holm [7]. All the analytes were eluted within 16 min at a column temperature lower than 130°C at the conditions used in this work. The column gave good resolution of the analytes from plasma components (see Fig. 3 and 4) without any sign of analyte decomposition checked by variation of the residence time. Adsorption losses of the mononitrates were too low to be detected from the calibration curves which were linear in the concentration range of interest (see Table II).

The pre-column used as the injector liner was cut off from another column of the same type and replaced daily after 35–40 injections. The coated pre-column appeared to be more inert compared with an uncoated siloxane deactivated column because more samples could be injected without peak-tailing of the mononitrates.

Accuracy and presision

The recovery was close to 100% when determined at the 4.5 and 50 ng/ml level and the intra-assay precision was within 1–4% R.S.D. (see Table I). The inter-assay precision was 6.6% (5.2 ng/ml ISMN-2), 3.7% (28 ng/ml ISMN-5) and 4.7% (1.2 ng/ml ISDN). The method detection limit at the 99% confidence level was determined as three times the standard deviation of the concentration at a low level (Table I). The detection limit was 0.4 ng/ml for the mononitrates and 0.2 ng/ml for ISDN but can be decreased further by concentration of the GPC fractions.

CONCLUSIONS

Isolation of drugs and other small molecules from complex plasma extracts by GPC could be of general value as a purification method prior to capillary GC. The GPC purification in this application made it possible to inject at least 50 samples without replacement of the injector liner. ISDN was hydrolyzed in whole blood and samples had to be cooled and centrifuged within 30 min after sampling.

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