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Short communication

Improved extraction procedure for the assay of isosorbide-5-mononitrate in serum

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Abstract

A procedure is described for the extraction of isosorbide-5-mononitrate from serum with diethyl ether–*n*-butyl acetate (15:1, v/v). After extraction, the diethyl ether is evaporated at room temperature and the nitrate remains dissolved in the relatively non-volatile *n*-butyl acetate phase. Glassware must be used throughout as the recovery of isosorbide-5-mononitrate is not reproducible when plastic tubes or plastic pipettes are used. The extraction recovery of isosorbide-5-mononitrate from serum is 67±7%, measured relative to external standards.

Keywords: Isosorbide-5-mononitrate

1. Introduction

Isosorbide-5-mononitrate (IS-5-MN) is an organic nitrate with vasodilating properties, used in the treatment of various cardiovascular diseases. IS-5-MN is also formed from isosorbide dinitrate which is used similarly as a vasodilator. The other metabolite formed from the dinitrate is isosorbide-2-mononitrate (IS-2-MN). For the determination of the mononitrates in blood, capillary gas–liquid chromatography with electron-capture detection (GC–ECD) is mostly used. GC–ECD offers high selectivity and sensitivity in the low ng-range [1–12].

For the extraction of the mononitrates from plasma or serum, liquid–liquid [1–9,13] and solid-phase [10–12] procedures have been developed. After extraction, the solutions are usually concentrated by evaporation of the solvent. Because of the volatility of the nitrates it is necessary to avoid complete evaporation of the solvent. In this paper we describe a liquid–liquid extraction/evaporation procedure which gives reproducible recoveries and with which the risk of accidental evaporation of the nitrates is greatly reduced. Although the method was developed for the analysis of IS-5-MN in blood, it is equally suitable for the analysis of IS-2-MN. During the development of the method we found that good results were obtained without silanizing of glassware, thereby making the determination of the mononitrates simpler than in previously published procedures.

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2. Experimental

2.1. Chemicals

The reference compounds IS-2-MN (purity 99.5%) and IS-5-MN (purity 99.9%) were obtained from Cedona Pharmaceuticals (Haarlem, Netherlands). The solvents used for the extraction of IS-5-MN from serum were of analytical grade.

2.2. Instrumentation and gas chromatographic conditions

The gas chromatograph used was a HP 5890 series II from Hewlett-Packard (Amstelveen, Netherlands) equipped with a HP 7673 automatic injector, a 15 mCi ^{63}Ni-electron capture detector, and a split–splitless injector with glass liner (8 cm×4 mm I.D.). The column was a fused-silica capillary column type DB 1701 (30 m×0.32 mm I.D.) with a 0.25-μm film thickness (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a flow-rate of 3 ml/min. The injector was operated in the split mode with a total helium flow of 100 ml/min. Nitrogen was used as the detector make-up gas at 40 ml/min. The injector and detector temperatures were held at 170°C. The column was maintained at 165°C. Before analysis, ten injections were made to sensitize the detector response. Peak heights were measured with a ChromJet integrator from Spectra-Physics (Eindhoven, Netherlands).

2.3. Preparation of standards

Stock (1 mg/ml) and working solutions of IS-2-MN and IS-5-MN were prepared in methanol and stored at −20°C. Working solutions were prepared once a week. Calibration graphs were obtained each day of analysis by spiking drug-free serum samples with known amounts of IS-5-MN (20–500 ng/ml). All samples were extracted and analyzed in the way described below.

2.4. Extraction procedure

To 1.0 ml of serum, 100 ng IS-2-MN was added as the internal standard. For extraction of the nitrates, 3.0 ml diethyl ether (saturated with water) and 0.2 ml *n*-butyl acetate were added. After vigorous shaking for 30 s the mixture was centrifuged for 5 min at 3000 *g* to separate the phases. The organic layer was transferred with a glass pipette into a glass tube and concentrated at room temperature under a gentle stream of nitrogen. After about 10 min, most of the ether was evaporated. The remaining solution was diluted with 0.8 ml *n*-butyl acetate and 1 μl of the resulting solution was injected into the gas chromatograph. Concentrations were calculated from peak-height ratios of IS-5-MN to the internal standard IS-2-MN.

2.5. Analytical parameters

Extraction recoveries were determined by comparing peak heights measured after extraction of spiked serum samples with the peak heights measured in external standards (50 to 500 ng/ml) prepared in diethyl ether–*n*-butyl acetate (15:1, v/v). Sample stability was studied by assaying stored (−20°C) serum standards (50 and 500 ng/ml) each day for a period of two weeks. Within-day precision and day-to-day precision were determined at four concentrations (20, 50, 200, 500 ng/ml) with spiked serum standards analyzed eight times (Table 1). Samples were stored at −20°C until analysis.

3. Results and discussion

Fig. 1 shows representative chromatograms obtained from a blank (A) and a patient serum sample (B). With the procedure described, the extraction recovery of IS-5-MN is 67±7% (n=6). This recovery is constant over the concentration range from 50 to 500 ng/ml. The diethyl ether is quickly evaporated and the *n*-butyl acetate remains in the test tube with the nitrates dissolved. When the extraction is carried out with diethyl ether alone and evaporation is continued until dryness, we found that the recovery was not reproducible. We also found that the recovery is irreproducibly decreased when plastic tubes or pipettes are used. Apparently, IS-5-MN adheres to plastic, and glassware should be used throughout. This was also reported by others [3].

Within-day and day-to-day precision (Table 1) are similar to those found by others [2,4–7,9–13]. The

Table 1
Precision and accuracy of the determination of isosorbide-5-mononitrate in serum

Amount added (ng/ml)	Amount measured (mean±S.D., $n=8$) (ng/ml)	C.V. (%)
Within-day variability		
20	18.8±1.6	8.4
50	44.1±3.7	8.5
200	190.5±7.2	3.8
500	512.9±18.4	3.6
Day-to-day variability		
20	18.7±5.7	30
50	46.7±4.9	10
200	191.4±8.1	4.2
500	492.3±15.7	3.2

lower limit of detection defined as the peak height equal to four times the signal-to-noise ratio of the detector is 10 ng/ml IS-5-MN when 1 ml of serum is extracted and 1 μl is injected as described in Section 2. The assay is linear up to at least 500 ng/ml and IS-5-MN is stable in serum for at least fourteen days when stored at −20°C. Reproducibility of the detector response is critically dependent on a clean injector glass liner [2,3,9,11]. With the present method, the detector response remains stable for at least 100 injections. Each day, however, the detector response must be sensitized by ca. ten repeated

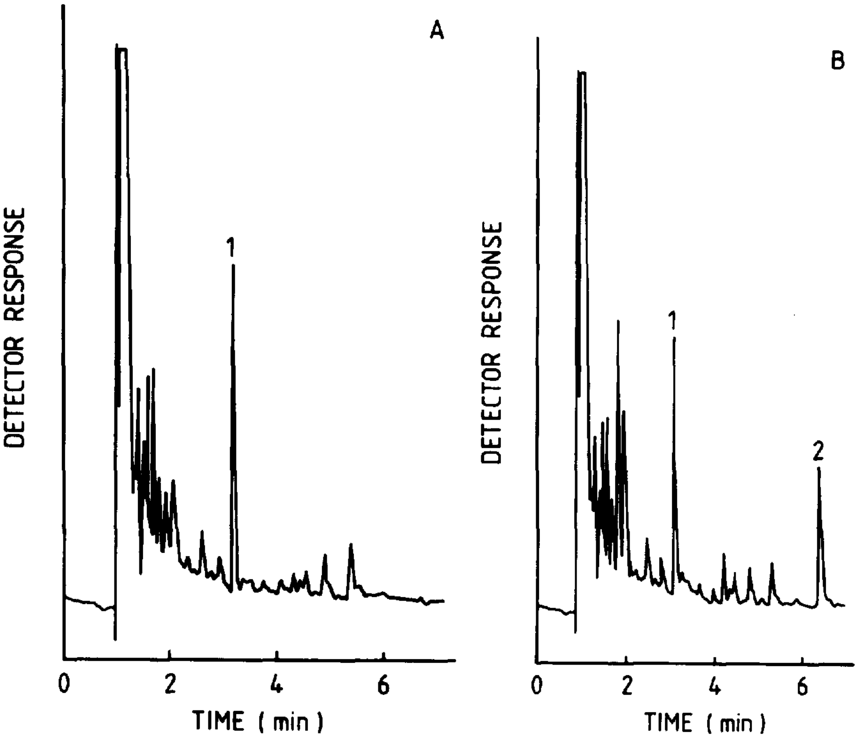

Fig. 1. The separation of the internal standard isosorbide-2-mononitrate (peak 1, retention time 3.2 min) and isosorbide-5-mononitrate (peak 2, retention time 6.5 min) on a fused-silica capillary column type DB 1701 (30 m×0.32 mm I.D.). Conditions are as described in Section 2. Chromatogram A is from a blank serum sample and chromatogram B is from a patient serum sample containing 227 ng/ml isosorbide-5-mononitrate.

injections of a standard prior to the sample injections. This has also been observed by others [3,14].

The advantage of this extraction procedure is that the time of evaporation is not critical. Diethyl ether is quickly removed in about 10 min and the remaining *n*-butyl acetate prevents the nitrates from evaporation. We observed a constant recovery of IS-5-MN from serum samples in non-silanized glassware. Silylation has not been tested, however, and it might be worthwhile to evaluate its effect on the analytical performance.

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