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IMPROVED METHOD FOR THE RAPID DETERMINATION OF ISOSORBIDE DINITRATE IN HUMAN PLASMA AND ITS APPLICATION IN PHARMACOKINETIC STUDIES

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SUMMARY

A rapid, accurate and highly sensitive method was developed for the determination of isosorbide dinitrate in human plasma. Concentrations in the lower nanogram and subnanogram range are determined by a one-step extraction of 2 ml plasma, containing 4 ng/ml nitroglycerine as internal standard, with 5.5 ml *n*-pentane. The extract is subjected to gas—liquid chromatography—electron capture detection analysis. The lower limit of quantitation is 200 pg/ml, but concentrations as low as 50 pg/ml are still detectable. The method allows the quantitative determination of isosorbide dinitrate plasma levels in man following a 5 mg sublingual administration up to four hours after application.

INTRODUCTION

Polynitric esters which are used as vasodilators in the therapy of angina pectoris, are still pharmacologically effective in doses of only a few milligrams. In view of the fact that these substances are very quickly metabolized [1, 2] and the blood levels of the unaltered drugs are within the lower nanogram and subnanogram range, their quantitative analysis has to meet high requirements with regard to the sensitivity of detection and accuracy of the method.

For the detection of isosorbide dinitrate (ISD) in human plasma after administration of therapeutic doses, gas—liquid chromatography (GLC) has proved to be adequate [3—8], in addition to the use of ^{14}C-labelled isosorbide dinitrate [2]. While sensitivity of detection is inadequate with flame ionization detection [3, 4], the detection limits are scaled down to the picogram range with electron capture detection [5—8]. When gas chromatographic (GC) analytical procedures are applied in this concentration range, the necessary elimination of interfering plasma compounds by a preceding treatment of the sample often leads to a compromise between the sensitivity as

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well as accuracy and time-consuming experimental procedures.

The ISD—electron capture detection methods which have been described up to now, either are highly sensitive and accurate, but very time-consuming [6], or they are, if less time-consuming, less sensitive [8] or less reproducible [7]. Other methods available are not sufficiently characterized to demonstrate their application in the lower nanogram range [5, 8]. The present work describes an electron capture—GLC determination of ISD which combines particularly high sensitivity and good reproducibility as well as accuracy with a very simple and quick procedure of analysis. The total time consumption for processing and GC procedure amounts to 20 min. The method therefore is particularly well suited for the routine determination of large quantities of samples.

The validity of the method described is demonstrated by the determination of ISD plasma levels in human volunteers after sublingual administration of an ISD tablet that allowed the evaluation of some pharmacokinetic parameters.

EXPERIMENTAL

Reagents and materials

Spectroscopic grade *n*-pentane (Merck, Darmstadt, G.F.R.) was three times glass-distilled, the third distillation being shortly before use. Spectroscopic grade *n*-hexane (Merck) was three times glass-distilled; a fourth distillation was performed after refluxing for 2 h over liquid Na-K-alloy. Nitroglycerine (Merck) was used as a 1% solution in ethanol. ISD was available as a 50% preparation on powdered lactose base (Schweizer Sprengstoffabrik, Dottikon, Switzerland). The nitrate was extracted with *n*-pentane and purified by recrystallization. To avoid contamination of blood plasma by plasticizers from plastic materials during sampling and processing [9] the nitrate-containing probes were allowed to contact only glassware during the whole analysis.

Extraction procedure

In a stoppered 20-ml centrifuge tube, 2 ml of plasma were spiked with 8 ng of nitroglycerine as internal standard by adding 10 μl of a 0.8 μg/ml solution of nitroglycerine in *n*-hexane and agitating for a few seconds. To this 5.5 ml *n*-pentane were added and the tube was shaken for 40 sec. Immediately after shaking and without centrifugation the organic layer was transferred to a conical glass tube and carefully evaporated at room temperature under a gentle stream of nitrogen. The nitrogen was washed by passing it through liquid paraffin. To prevent evaporation of nitroglycerine the nitrogen stream was interrupted as soon as dryness was achieved. After addition of 100 μl of *n*-hexane to the residue, the solution was agitated for a few seconds and the samples were stored at 4° until injection into the gas chromatograph.

Electron capture GLC

A Packard Model 419 gas-chromatograph equipped with a 10 mCi ^{63}Ni electron capture detector (ECD) was used.

Argon—methane (95:5) was used as carrier gas with a flow-rate of 20 ml/min

through the column and as scavenger gas with a flow-rate of 35 ml/min through the detector. The gas was dried over a molecular sieve (Oxisorb F, WGA, Düsseldorf, G.F.R.). Installation of flow-controlling gas valves (DC 1400, Kontron, Munich, G.F.R.) instead of pressure-controlling valves resulted in a striking reduction of the baseline noise. Maximum sensitivity was obtained with the following temperature settings: oven 130°, injection port 165°, detector 180°. Under these conditions retention times were 50 sec and 165 sec for nitroglycerine and ISD, respectively. The detector was operated with an electron capture lineariser (Model 736, Packard) in the "constant current pulse mode" with a reference current of $1.75 \cdot 10^{-9}$ A.

The septum of the injector port was changed every 20 injections. The septum was pretreated by washings with *n*-hexane, followed by drying at 200° for 2 h. The injection volume was 4 µl and the injection was performed in duplicate. For injection, a 5 µl glass syringe (SGE, Melbourne, Australia) was used.

Quantitation

Quantitation of ISD was achieved by using nitroglycerine as internal standard. The peak heights of the two nitrates on the chromatogram were measured. From the ratio of the peak heights, obtained by analysing plasma samples to which were added known amounts of nitroglycerine and ISD, a calibration curve was constructed. To eliminate the influence of possible long time gradual variations of the calibration factor, the calibration was performed weekly.

Column

The glass columns, 1.2 m × 2.4 mm I.D., were silanized by treatment with a solution of 1% trimethylsilylchloride (Merck) in benzene for 1 h. After flushing with methanol, the column was air-dried and loaded with 3% silicone OV-101 on Gas-Chrom Q, 80—100 mesh (WGA). The column was primed before use by heating (100°) for 2 h under nitrogen flow, subsequent thermal aging at 340° without nitrogen flushing (no-flow-conditioning) overnight, and conditioning with carrier gas flow for 24 h at 270°. Priming without no-flow-conditioning could also be achieved, but in this case it takes over a week for the full sensitivity to be attained.

Plasma level study

Eight healthy volunteers (female, aged 18—50 years) received a 5-mg sublingual ISD tablet*. Before, as well as 6, 11, 16, 23, 31, 46, 61, 90, 120, 180 and 240 min after administration, ca. 10 ml blood were drawn from the cubital vein into heparinized glass syringes. In order to prevent further metabolism or disintegration of ISD in vitro, the blood was transferred from the withdrawal syringe into iced glass tubes (where 50 µl of 0.002 *N* silver nitrate solution was added) and immediately centrifuged at 1300 *g* in a refrigerated centrifuge at 4°. Addition of larger amounts of silver nitrate resulted in hemolysis. The plasma was either processed immediately for further analysis

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or deep frozen until the analysis was to take place (not later than 8 h after withdrawal of the sample).

RESULTS AND DISCUSSION

Sensitivity

With the described procedure chromatograms were obtained in which ISD and nitroglycerine appeared as symmetrical peaks which were hardly influenced by other plasma components or impurities. Fig. 1A shows a typical gas chromatogram of the nitrates extracted from human plasma. Fig. 1B shows a chromatogram of blank plasma from the same subject treated by the same procedure. The recording conditions for both chromatograms were: attenuation 1 X 64, 5 mV recorder operated at a charge speed of 600 mm/h.

Corresponding to a recording amplitude of 200 mm the signal heights of ISD and nitroglycerine are 150 mm and 170 mm respectively. The concentration of ISD was 5 ng/ml in plasma and that of nitroglycerine 4 ng/ml in plasma. This corresponds to amounts of 400 pg and 320 pg injected onto the column. The minimum detectable amount of ISD as a solution of the pure substance, was 2 pg (signal-to-noise ratio = 3:1).

Based on the extraction of 2 ml plasma the lower limit of detection of ISD in plasma was 50 pg/ml. In some cases ISD-free plasma showed "isosorbide dinitrate" peaks, which, however, never corresponded to more than 100 pg/ml. A plasma concentration of 200 pg/ml therefore was regarded as the lowest limit of quantitative determination.

A lowering of the GC response for nitroglycerine but not for ISD was observed, when more than approx. 45 min elapsed between two injections.

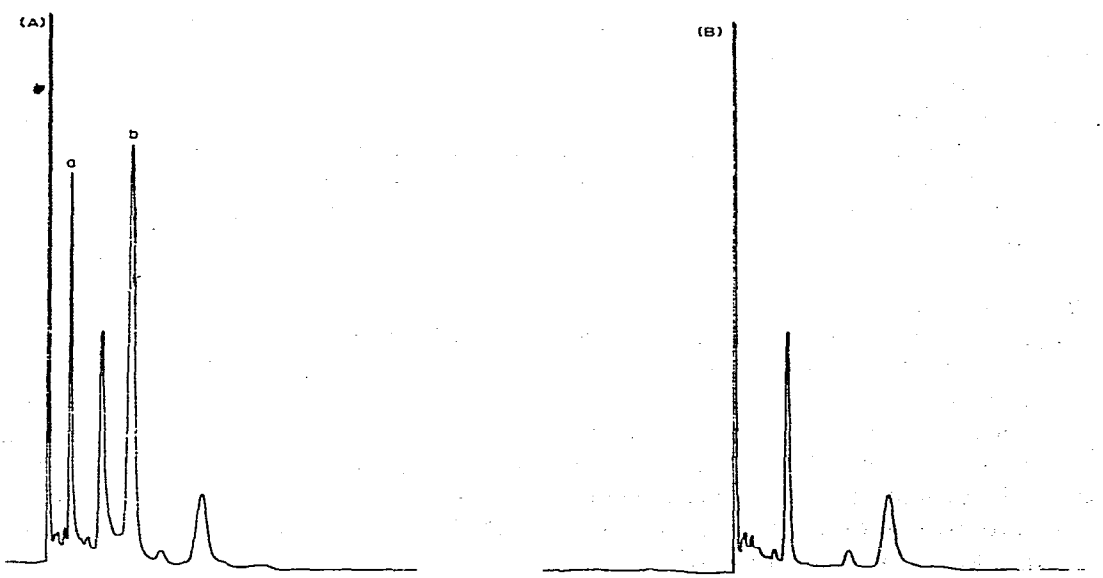

Fig. 1. Typical chromatograms obtained from human plasma extracts: A, plasma spiked with 4 ng/ml nitroglycerine and 5 ng/ml ISD. Peaks: a = nitroglycerine, b = ISD. B, blank plasma.

In this case full sensitivity could be re-established by two or three 1-μl injections of a 1-μg/ml solution of nitroglycerine. The lack of memory effects could be proven by injection of pure solvent following the injection of nitrates.

Exhaustion of the column was indicated by a loss of sensitivity for both nitrates and was observed after approx. 2000 injections.

Accuracy, reproducibility

Calibration curves were constructed by analysing samples to which different amounts of ISD were added to yield concentrations of 0.5, 1.0, 2.0, 5.0, 10.0 and 15.0 ng/ml of plasma. The concentration of the internal standard nitroglycerine was held constant (4 ng/ml). A linear relationship between the response ratios of the two nitrates and ISD concentrations was observed. Table I shows the measured response ratios of five calibration curves obtained during five different weeks. The coefficients of a linear regression analysis of the calibration values were found to be in the range from 0.9966 to 0.9998,

TABLE I

RATIOS OF THE PEAK HEIGHTS OF ISD AND NITROGLYCERINE

Obtained from plasma runs over several weeks.

ISD added (ng/ml)	Ratio of peak heights Weeks				
	1	2	3	4	5
0.5	0.036	0.042	0.024	0.052	0.049
1.0	0.082	0.069	0.066	0.097	0.095
2.0	0.159	0.165	0.143	0.179	0.195
5.0	0.451	0.456	0.426	0.444	0.541
10.0	0.996	0.892	0.962	0.915	1.034
15.0	1.428	1.398	1.336	1.365	1.550

TABLE II

REPRODUCIBILITY OF ISD DETERMINATION BASED ON SUCCESSIVE PLASMA RUNS

ISD plasma concentrations

Given (ng/ml)	Found Individual plasma runs (ng/ml)					Average (ng/ml)	Coefficient of variation (%)
1.0	0.88	1.05	0.83			0.92	13
1.0	0.89	0.95	0.98	1.04	0.91	0.95	6
2.0	2.28	2.18	2.33			2.26	3
4.0	4.83	4.50	4.80			4.71	4
5.0	5.01	5.81	5.45	5.25	5.35	5.37	5
10.0	9.96	11.36	10.27			10.53	7
15.0	16.43	14.98	15.44	15.64	15.02	15.50	4

thus indicating the high accuracy of the concentrations determined and the good linearity of the calibration curves. The reproducibility of the analysis as determined by repeated assays of plasma with known ISD concentrations is shown in Table II. Triplicate and quintuplicate assays were carried out in different months. The highest coefficient of variation found at a concentration of 1 ng/ml was 13%, corresponding to a standard deviation of ± 120 pg ISD per ml of plasma. Considering that assays reported previously involve coefficients of variation up to 50% in this lower concentration range, this result means a substantial improvement of the reliability of the measured values.

Recoveries

Total recoveries of nitroglycerine and ISD including all steps of the analytical procedure were determined by a comparison of the peak heights obtained from processed plasma samples with the peak heights of directly injected standards. Plasma samples with known ISD concentrations from 0.5 ng/ml to 10 ng/ml and nitroglycerine concentrations of 4 ng/ml as well as standard solutions of 100 ng/ml in *n*-pentane were used. The results (Table III) implicate that nitroglycerine is somewhat more readily extracted than ISD. However, there is no concentration-dependent trend in the recoveries observed. Variations between individual recovery values are mainly attributed to different volumes of *n*-pentane solution obtained by separating the organic layer from the aqueous phase. In addition the given recoveries also include accidental errors by variations in GC response.

Duration of assay

One of the goals of this work was to reduce as much as possible the time needed for ISD assays. Some characteristics of the described assay make it possible to dispense with time consuming purification procedures without obtaining interfering peaks in the chromatogram. The choice and high purity of the solvents *n*-pentane and *n*-hexane are the most essential characteristics of our method. The homopolar character of *n*-pentane and the short extrac-

TABLE III

TOTAL RECOVERY OF ISD AND NITROGLYCERINE ADDED TO HUMAN PLASMA

n = number of conducted analyses

	Amount added (ng/ml)	*n*	Recovery (%) (average ± S.D.)	Recovery (%) (total mean ± S.D.)
Isosorbide dinitrate	0.5	3	62 ± 9	
	1.0	3	71 ± 10	67 ± 10
	2.0	3	66 ± 4	
	4.0	3	65 ± 14	
	7.0	3	71 ± 7	
	10.0	3	71 ± 18	84 ± 18
Nitroglycerine	4.0	12	84 ± 18	

tion time of 40 sec prevent the formation of emulsions thus making it unnecessary to separate the phases by centrifugation. A further advantage of *n*-pentane is its rapid evaporation. As we use a small *n*-pentane volume of 5.5 ml for extraction, the total time for plasma processing can be reduced to 12 min. A comparison of chromatograms of processed plasma with and without added nitroglycerine, revealed that already a slight modification of the solvent properties can modify the result: the trace amount (0.8 μg) of ethanol derived from the undiluted nitroglycerine stock solution added by spiking the plasma with the internal standard, caused a remarkable increase of a peak with the retention time of 111 sec (largest peak in Fig. 1B) that could not be attributed to ethanol itself. The same increase was observed when we added 1 μg of ethanol to 2 ml of plasma prior to the extraction as performed to obtain the chromatogram of Fig. 1B.

Furthermore, the time needed for the assay would have been reduced by concentrating the *n*-pentane volume to 100 μl instead of evaporating it to dryness. But when such a solution was injected into the gas chromatograph we obtained a lot of additional peaks that partially interfered with the nitrate peaks. As the solvent properties of *n*-pentane and *n*-hexane are only slightly different, we suppose that the dried residue on evaporation is partially irreversibly adsorbed at the glass surface of the tube. Presumably this desirable effect will not occur when the glassware is silanized [6].

As 8 min are needed for the GC procedure, about 20 min are required for a complete ISD analysis. There are no delayed peaks in the chromatogram, as has been observed in previously reported assays [6]. Therefore, every 8 min another sample can be injected.

In vivo studies

Table IV shows the results of ISD plasma level determinations in human volunteers according to the method described. All plasma values were obtained by duplicate assays. The sensitivity of the method allowed detection of ISD

TABLE IV

PLASMA CONCENTRATIONS OF ISD (ng/ml) FOLLOWING A 5 mg SUBLINGUAL DOSE

N.D. = not detected

Time after dosing (min)	Subject								Mean ± S.D.
	1	2	3	4	5	6	7	8	
6	6.3	N.D.	0.8	7.6	13.7	1.0	1.9	2.5	4.2 ± 4.7
11	16.8	34.9	8.1	17.0	27.6	4.2	10.7	13.4	16.6 ± 10.2
16	15.5	42.1	5.2	13.1	—	5.0	10.5	29.0	17.3 ± 13.5
23	13.0	24.3	8.0	11.0	15.0	5.7	11.2	22.1	13.8 ± 6.5
31	10.1	18.6	6.7	8.5	13.5	5.2	9.7	16.9	11.2 ± 4.8
46	6.3	10.5	2.9	5.4	10.5	4.9	6.3	11.1	7.2 ± 3.1
61	4.2	8.0	3.0	8.6	5.7	3.7	9.5	5.8	6.1 ± 2.4
91	1.8	3.8	1.4	4.0	2.9	1.4	3.0	3.2	2.7 ± 1.0
120	0.9	1.9	0.3	1.2	1.4	0.7	0.9	2.8	1.3 ± 0.8
180	0.3	1.1	N.D.	N.D.	0.5	0.3	0.4	0.7	0.4 ± 0.4
240	0.2	0.3	N.D.	N.D.	0.3	0.3	0.2	0.2	0.2 ± 0.1

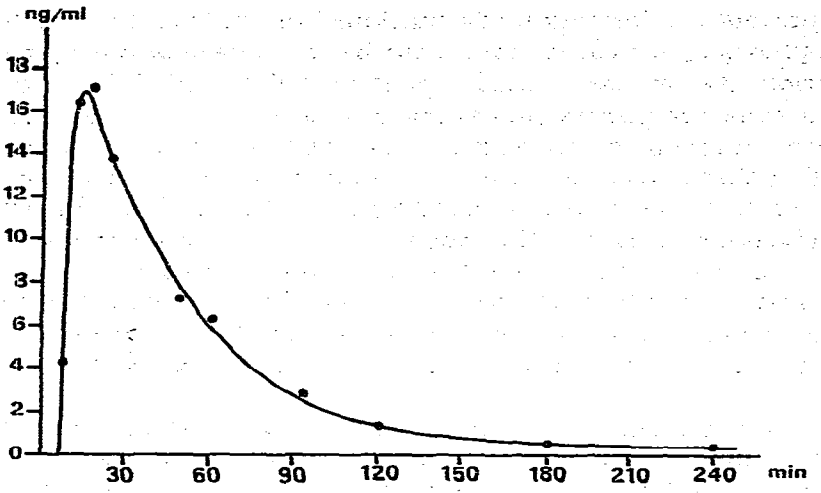

Fig. 2. Average plasma levels of ISD in 8 subjects after sublingual administration of a 5 mg tablet. The curve was calculated based on one compartment body model assumption.

in plasma even 3—4 h after ingestion of the tablet. The average plasma levels of all 8 volunteers are depicted in Fig. 2. ISD concentrations reached a maximum of 17.3 ng/ml within 16 min of administration. A pharmacokinetic one-compartment analysis of the average plasma level curve resulted in a mean half-life of 1.4 min for the absorption and 30.1 min for elimination of ISD. A lapse of approx. 5 min from ingestion of the tablet to the first detectable occurrence of ISD was calculated.

CONCLUSION

A rapid and accurate method for the determination of ISD in human plasma in the lower nanogram and subnanogram range was developed. The method, utilizing nitroglycerine as internal standard, is based on GLC analysis with a ^{63}Ni ECD. ECD assays of ISD have been reported previously. They either involved time-consuming multistep processing of the samples or a decreased accuracy in the lower nanogram range.

This assay combines a highly accurate and sensitive detection of ISD with a rapid experimental procedure. The sample processing is limited to essentially one step, carried out in 12 min. Total analysis including GC procedure can be performed in approx. 20 min.

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