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Urinary concentrations of threshold substances: a clear enough discrimination between doping and therapy? The case of ephedrines

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Introduction

Threshold substances are a delicate issue in Antidoping analysis. Especially for ephedrines, banned only “in competition” an inter-individual variability in metabolism and excretion can lead to sanction or not sanction the same behavior in different individuals.

The use of these drugs, especially pseudoephedrine, is widespread among athletes, due to its decongestant action, but also for its stimulant activity. We noted an increase in the number of samples presenting with an elevated concentration (100 μg/mL and higher) of pseudoephedrine both in particular periods of the year, probably due to the typical seasonal diseases (allergic rhinitis, influenza), and, also, in correspondence of specific sport competitions. During the Winter 2006 Olympic Games, 3 positive cases were reported for cathine (exceeding the cut-off values). At the same time, the presence of cathine was also detected in several other samples at a concentration not exceeding 5 μg/mL (and therefore not reported as adverse analytical findings), all of them with very high concentrations of pseudoephedrine (> 300 μg/mL). The same trend was also observed in the following months, with an increasing number of cases with high concentrations of pseudoephedrine (above 50 μg/mL), with cathine either exceeding or less than the cut off. Due to the scarce literature data on this topic (1-5), we performed an observational study on 9 healthy volunteers taking different doses of over-the counter preparations containing pseudoephedrine for self-medication. At first, a method for quali-quantitative determination of ephedrines in urine was set up and fully validated, by an implementation of the method described by Forsdahl and Gmeiner (6), using as internal standards ephedrine-D3. The method has then been applied to observe the variability of concentrations of ephedrines/metabolites found in the urine samples

of subjects taking these medications in therapeutic doses for self-medication (for the treatment of allergic rhinitis or against influenza symptoms) and, if so, at what administered dose the threshold value was exceeded, to verify whether a "population" threshold could discriminate between the administration of ephedrines for therapeutic uses and their administration with the aim of improving sport performance.

Oral fluid is considered a useful diagnostic tool for the determination of early drugs consumption, being the presence, and in some cases also the concentration, of xenobiotics in this biological fluid directly related to the corresponding picture in plasma, mainly in terms of appearance/disappearance of the drugs (7-11). Saliva can hence help to evaluate if the person is still under the effect of the substance at the time of sampling, important issue for substance prohibited only "in competition".

In parallel to urine, the subjects involved in the study were hence required to give also oral fluid (OF) specimens, sampled every 1.5-2 hours.

Materials and Methods

Chemicals and Reagents

Ephedrine-D_3 and cathine were obtained from LGC Standards (Milano, Italy); ephedrine, pseudoephedrine, phenylpropanolamine (norephedrine), N-methyl,N-trimethylsylil trifluoroacetamide (MSTFA), tert-buthyl-methyl ether, sodium hydroxide, trimethylchlorosylane were supplied by Sigma-Aldrich (Milano, Italy); Actifed and Reactine were purchased from Pfizer Consumer Health Care, (Latina, Italy), Fienamina was from Recordati, Milano, Italy.

Excretion studies

All the subjects gave informed consent prior to participate to the study and were submitted to medical evaluation. Excretion studies on pseudoephedrine were performed on 9 subjects, 4 males (age: 23-41; weight: 76-95 Kg) and 5 females (age: 24-41; weight: 42-55 Kg) taking either Actifed (pseudoephedrine 60 mg, triprolidine 2.5 mg), or reactine (pseudoephedrine 120 mg, cetirizine 5 mg, in a sustained-release formulation). Ephedrine excretion studies were performed on three subjects, that took a single dose of Fienamine (ephedrine 12 mg, chlorfenamine 10 mg, sustained release). All the subjects were followed after the administration of one single dose of pseudoephedrine (60 mg each). Seven subjects also took a double dose (120 mg) of pseudoephedrine; one subject took also 180 mg of

pseudoephedrine in a single dose and two doses of 120 mg each with a delay of 12 hours; the same subject took also two doses of pseudoephedrine (120 mg) five times. One subject took three doses of 60 mg with a delay of 12 hours between each other. In each study a blank urine sample was collected immediately prior to drug administration; then all urines produced in the first 12 hours, and at least the first and last urine of the second day, were collected. Urine samples were collected in pharmaceutical reservoirs, pH values were measured with pH indicator strips and then stored at –20°C until analysis. Specific gravity was measured with a RE50 refractometer from Mettler Toledo (Milano, Italy) and creatinine by a 100 scan UV spectrophotometer from Varian (Torino, Italy) at 492 nm, after reaction with picric acid.

In each study a control urine and oral fluid samples were collected immediately prior to drug administration, followed by all urine produced in the first 12 hours and at least the first and last urine of the second day; oral fluid samples were collected every 2 hours for 24 hours, except during night, using Salivette devices (Sarstedt, Germany).

Calibration curves

Methanolic standard stock solution of the substances of interest were prepared at a concentration of 1 mg/mL for urine analysis and of 1 µg/mL for oral fluid, by diluting the reference solutions in methanol, and stored at –20°C.

Calibration curves were prepared by addition of the appropriate amount of ephedrine, cathine, pseudoephedrine and norephedrine to 1 mL of blank urine or oral fluid to obtain the following concentrations: 2.5, 5, 10, 15, 25, 50, 100, 200 µg/mL for urine and 2.5, 5, 10, 25, 50, 100, 500 ng/mL for OF.

Sample preparation

To 1 mL of sample was added 25 µL of ephedrine-D3 (1 µg/mL for oral fluid and 0.1 mg/mL for urine) and the mixture was alkalynised by addition of two drops of sodium hydroxide 1M. To this was added 200 mg of sodium chloride and the mixture was extracted with 2 mL of tert-buthyl-methyl ether. The organic layer was separated and 200 mg of anhydrous sodium sulfate added to remove any residual water, vortexed, transferred in another vial, dried under gentle stream of nitrogen at room temperature and derivatised with 50 µL of MSTFA/TMCS (1%) at 70°C for 30 min. The derivatised extract was injected directly into the GC/MS.

GC/MS conditions

The GC/MS system was an Agilent HP6890 gas chromatographer coupled to a 5973 mass spectrometric detector. Chromatographic conditions were the following: Supelco

custom-made 5% phenyl-methylsilicone capillary column (17m x 0.2 mm i.d., 0.33 μm film thickness). The oven temperature was held at 130°C for 1 min, increased to 200 at 8°C/min., increased to 280°C at 40°C/min (held 2 min.). The injection port was set at 270°C in split mode (split ratio 20/1) for urine analysis and in splitless mode for oral fluid, and helium was used as carrier gas at a constant pressure of 20 psi.

The mass detector operated in electron impact ionization at 70 eV in scan mode for urine analysis (scan range from 47 to 400) and in SIM mode for oral fluid analysis. Ions selected were at m/z 116, **117**, 163, 280 for cathine, 119, **120**, 283 for norephedrine-D_3, 130, **131**, 220, 294 for ephedrine and 133, **134**, 223, 297 for ephedrine-D_3 (underlined ions were used for quantification of ephedrines in saliva and those in bold for urine).

The method has been validated taking into consideration the following parameters: limit of detection and of quantification, specificity, linearity, intra and inter-assay accuracy and repeatability (precision).

Results and Discussion

Results obtained from excretion studies show high interindividual differences in the urinary concentrations of both pseudoephedrine and cathine, not depending on the weight, nor on the sex nor, in some instances, on the administered dose. The same typical therapeutic dose of pseudoephedrine (60 mg) produced a urinary concentration of over 5 μg/mL for cathine and of over 100 μg/mL in two out of nine subjects only. When a dose of 120 mg was administered, cathine concentration exceeded 5 μg/mL in four out of seven subject, also with levels of pseudoephedrine above 100 μg/mL. After administration of 5x120 mg of pseudoephedrine (120 mg administered every seven days for five weeks) to one of the subjects, the urinary concentration of cathine and pseudoephedrine exceeded 5 μg/mL (peak concentration: 14.8 μgm L^{-1}) and 100 μg/mL (peak concentration: 275 μg/mL) respectively; while when the same subject took 180 mg of pseudoephedrine the urinary concentration values were below 5 μg/mL for ephedrine and 100 μg/mL for pseudoephedrine. In order to evaluate how long the subjects were effectively under the effect of the drug, 8 subjects involved in the study collected also oral fluid samples during the first 12 hour after drug administration (oral fluid reflects with a good approximation plasma concentrations and times of appearance/disappearance of drugs). As for urine samples, results obtained from oral fluid

showed high inter- and intra-individual variability, in terms of concentrations of pseudoephedrine following the administration of the same dose, demonstrating different kinetics of absorption case to case. Times of appearance/disappearance are on the contrary more reproducible, and in all subjects pseudoephedrine was undetectable in oral fluid samples after 12 hours from administration; whereas urine samples, analyzed in parallel, show higher ephedrines concentrations, exceeding cut-off values, generally between 8 and 24 hours after administration of the drug. Only in the case of sustained-release formulations constant pseudoephedrine concentrations are achieved in oral fluid. These results are shown in figures 1- 6. In the case of ephedrine administration, two out of three subjects exceeded the 10 µg/ml threshold after a therapeutic dose (12 mg, sustained-release formulation) (Fig. 7).

The results obtained confirm a high inter-individual variability in the urinary concentration of pseudoephedrine and cathine following the administration of the same therapeutic dose of preparation. The 5 µg/ml threshold for cathine can easily be exceeded after administration of therapeutic doses of pseudoephedrine in some subjects; in the same way, the proposed value of 100 µg/ml for pseudoephedrine can be exceeded. Furthermore, in many cases, the cut-off values are exceeded 8-12 hours after administration, when the substances are not more detectable in the corresponding oral fluid samples suggesting their not recent use.

References

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Fig. 1. oral application of 60 mg of pseudoephedrine, time dependence of urinary concentrations of 9 subjects.

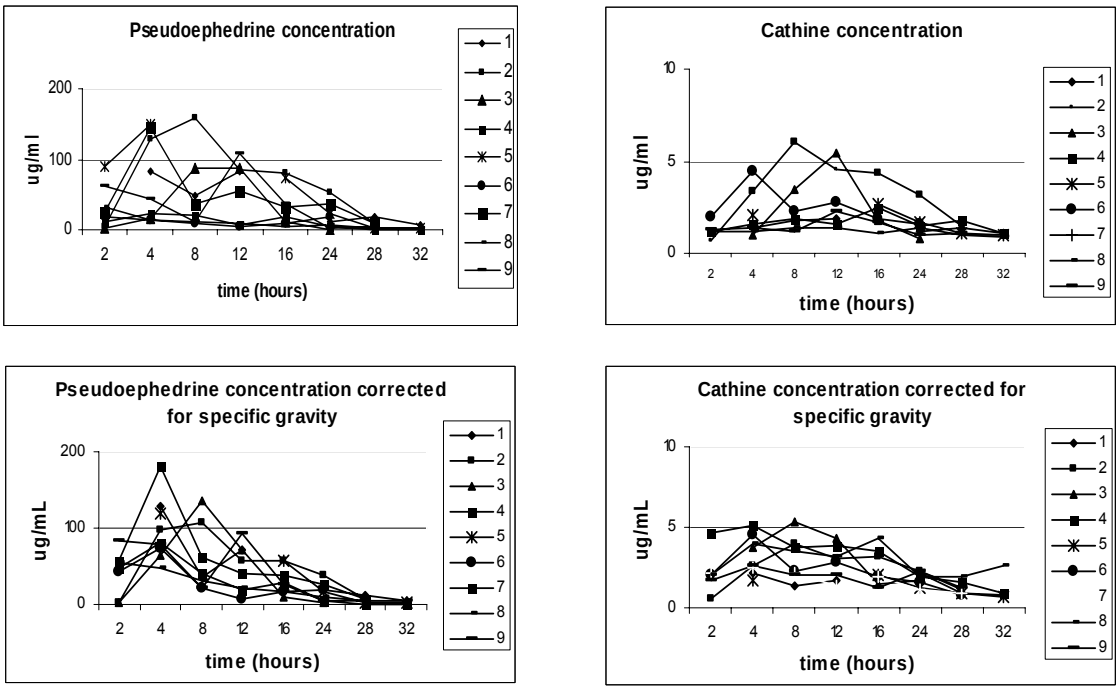

Fig. 2. oral application of 120 mg of pseudoephedrine, time dependence of urinary concentrations of 7 subjects.

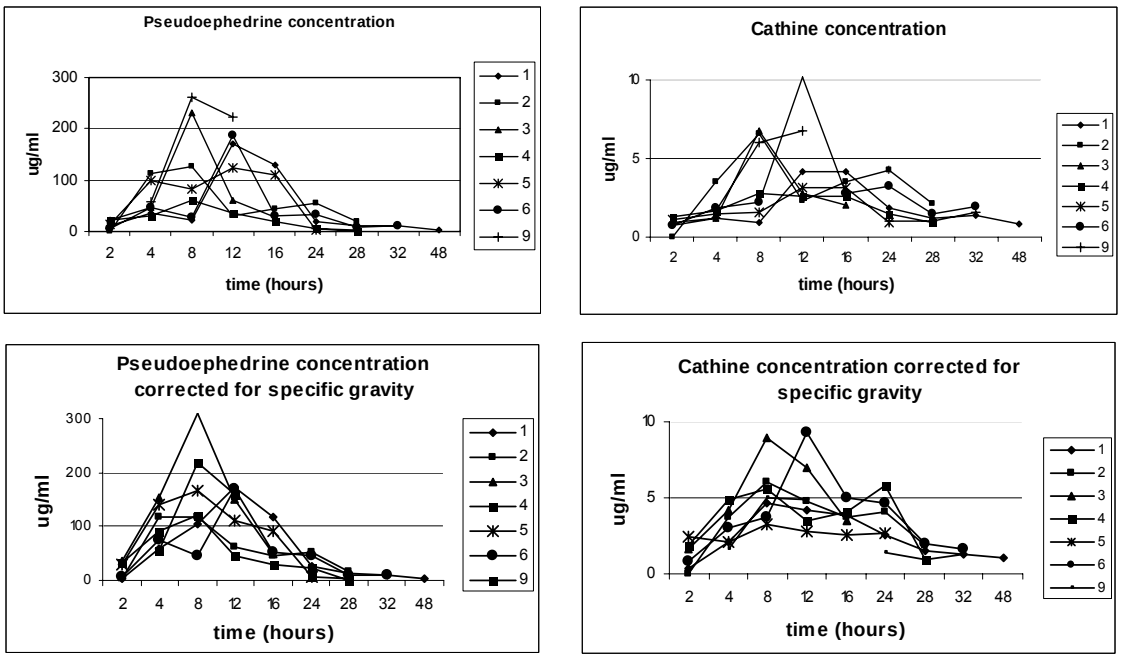

Fig. 3. Intra-individual study: oral application of 120 mg pseudoephedrine, urinary concentrations after five administrations to the same subject

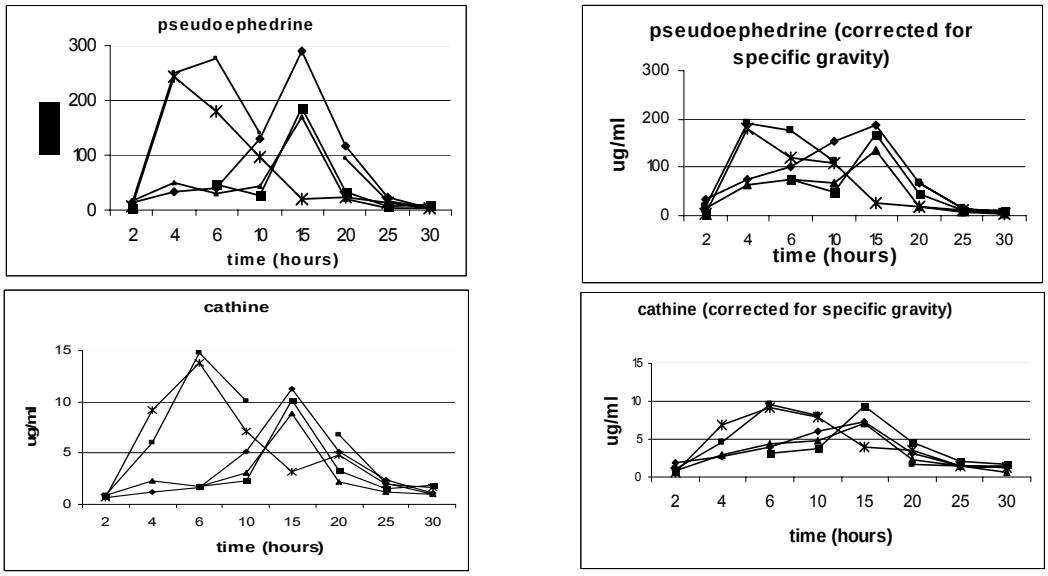

Fig. 4. Intra-individual study: 60, 120, 180, 2x120 mg administrations of pseudoephedrine to the same subject.

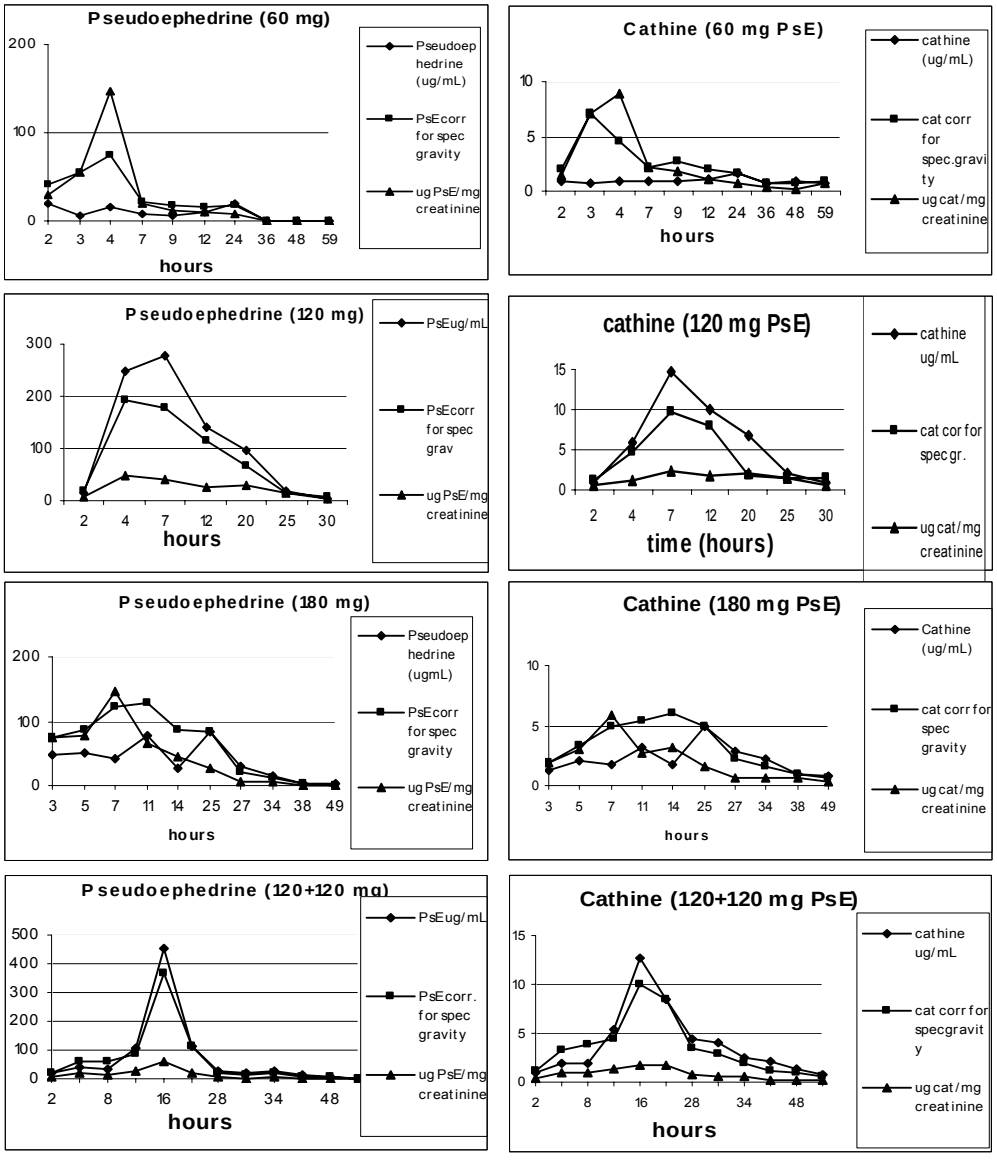

Fig. 5. Oral fluid results: concentrations of pseudoephedrine after oral application of 60 and 120 mg of pseudoephedrine (9 and 4 subjects, respectively)

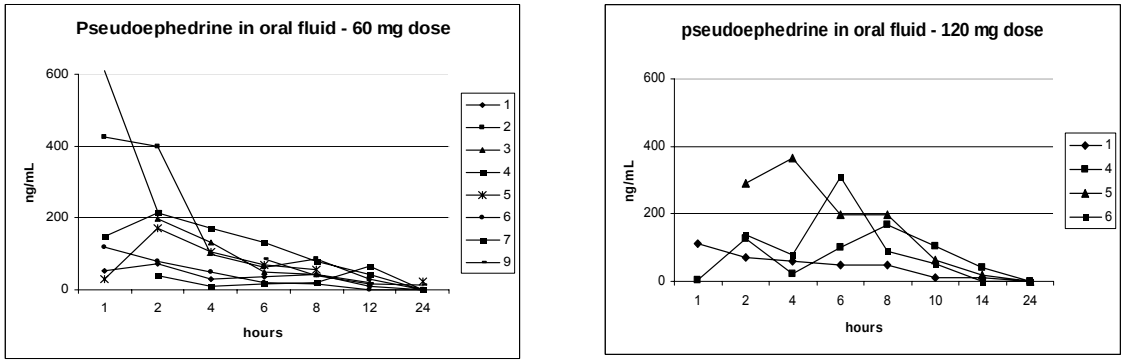

Fig. 6. Sustained release: oral administration of 120 mg of pseudoephedrine (one subject)

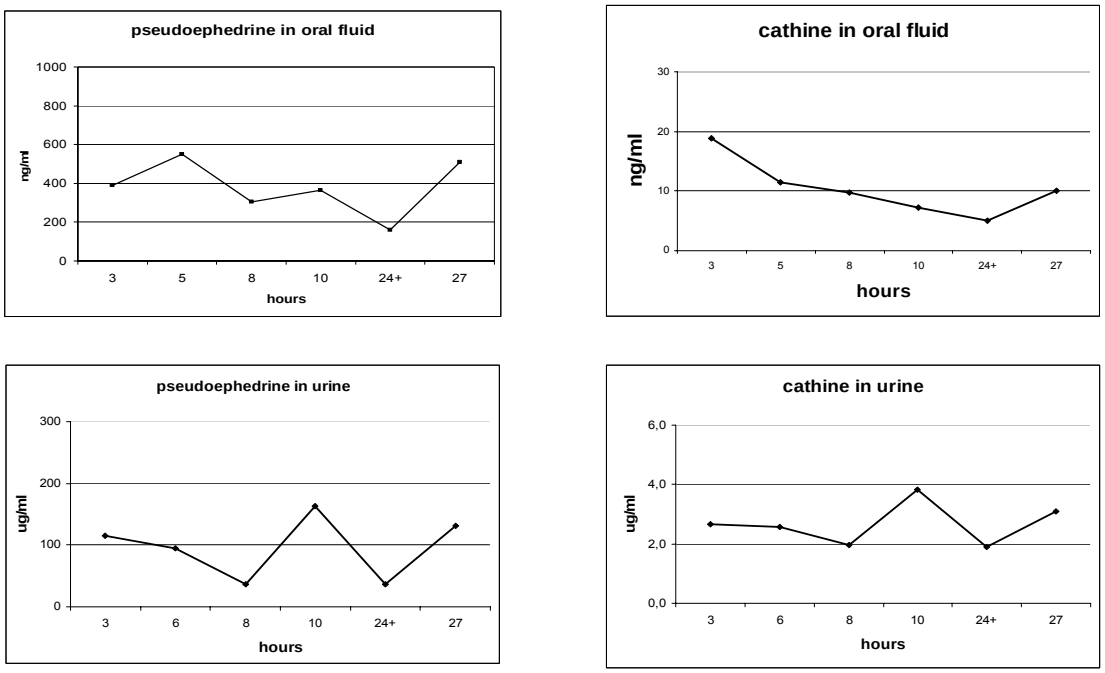

Fig. 7. Sustained release: oral administration of ephedrine 12 mg (3 subjects)