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Letter to the Editor

β-Glucuronidase from *Helix pomatia* origin is not suitable for diosmetin analysis

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Campanero et al. [1] have described an HPLC–MS/MS assay for the indirect analysis of diosmin and one of its metabolite, diosmetin in human plasma. The concentrations of this metabolite were measured after hydrolysis of putative glucuronides of diosmetin with β-glucuronidase from *Helix pomatia* origin. However, these authors have reported concentrations of diosmetin 50–500 times higher than all concentrations previously measured in our clinical studies with Daflon® 500 mg and we would like to report some recent findings on the risk of using *Helix pomatia* enzyme when analysing drugs of natural origin such as Daflon® 500 mg.

Whilst *Helix pomatia* β-glucuronidase is frequently utilised as a crude hydrolytic extract by metabolism scientists, it is also known as a potential source of contamination when analysing natural products. Its composition has been investigated by mass spectrometry analysis and some authors [2] have identified and quantified different endogenous flavone derivatives as well as phytoestrogens by MS/MS analysis.

In order to clarify whether the discrepancies between the different diosmetin levels reported in the literature and our internal data could be related to the source of hydrolytic enzyme used, we have investigated the analysis of β-glucuronidase from different origins and purification grades by UPLC–MS/MS techniques.

Our first studies have confirmed the presence of different flavonoid type of components in the β-glucuronidase extracts of *Helix pomatia* origin. Amongst all these flavonoids, a major analytical interference at the retention time of diosmetin, with the same multiple reaction monitoring transitions was observed both in the ESI^- (299 > 284) and $APCI^+$ (301 > 286) modes. A complementary structural analysis of this interference using high resolution mass spectrometry (Thermo Orbitrap XL model, 60 000 resolution, full width at half maximum at *m*/*z* 400) has allowed to unequivocally identify this interference being endogenous diosmetin by MS full scan analysis (same retention time and elemental composition determination), as well as identical collision induced dissociation fragmentation spectra obtained for the interference and for the reference diosmetin solution in both negative and positive modes.

The UPLC–MS/MS analysis of different batches of *Helix pomatia* β-glucuronidase (2 batches of non-purified H2 and 2 batches of purified enzyme by a sterilizing filtration procedure HP-2S, all obtained from Sigma) for their endogenous diosmetin content, has shown these concentrations were ranging from 1300 ng/ml up to 10 μg/ml.

In order to document the different circulating entities in plasma after single oral administration of Daflon® 500 mg to human healthy volunteers, we have developed three analytical assays:

- The first one, as a more metabolism orientated method for the simultaneous bio-analysis of diosmin, diosmetin and also its 3 potential glucuronides: 3′-β-D-glucuronide, 7-β-D-glucuronide, 3′,7-β-D-diglucuronide, using synthesized references of these glucuronide derivatives.
- The second method, focussing on the sensitive and rapid analysis of total diosmetin obtained after sample hydrolysis with β-glucuronidase. For this second assay, an enzyme from *Escherichia coli* origin, free on any flavonoid interference in our UPLC–MS/MS conditions, was used. This enzyme has proved to be free of endogenous diosmetin and therefore suitable for the analysis of diosmetin in plasma samples, down to the 0.1 ng/ml concentration level.
- The third assay corresponded to the method described by Campanero at al we have scrupulously reproduced, only adapted for the *E. coli* origin of β-glucuronidase instead of *Helix pomatia*.

These three assays were applied to the analysis of clinical samples obtained after single oral administration of Daflon® 500 mg to 12 human healthy volunteers. The concentrations of diosmin were below the limit of quantification, whilst very low concentrations of plasma circulating diosmetin were measured, in the range of the detection limit (0.1 ng/ml) with the specific assay. Moreover, two out of three glucuronide metabolites (3′-β monoglucuronide and 3′,7-β diglucuronide) have been detected in the biological media (plasma and urine) analysed, but only the 3′-β-D-glucuronide of diosmetin has been quantified with maximal plasma concentrations of 20 ng/ml at Cmax (see Table 1 for details).

All three methods were totally correlated for the diosmetin concentrations measured in these clinical samples and more importantly for us, totally coherent with historical data of diosmetin obtained after oral administration of Daflon® 500 mg.

Conclusion

The use of β-glucuronidase for glucuronide hydrolysis is a common practice in bio-analytical techniques. However, the presence of endogenous flavonoids and other related phytoestrogens in *Helix pomatia* preparations should be carefully investigated before its use as a hydrolytic enzyme in bio-analytical assays to measure flavonoids. As an example, we have unequivocally identified μg/ml concentrations of endogenous diosmetin in several batches of *Helix pomatia* β-glucuronidase, prohibiting the use of this enzyme at least for the analysis of diosmetin and related metabolites in plasma samples at the ng/ml range.

The diosmetin concentrations measured in our clinical study with either a specific assay of diosmin, diosmetin and its syn-

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Table 1
Examples of concentrations of diosmin metabolites measured after single oral administration of Daflon® 500 mg in healthy volunteers, by 3 different LC–MS/MS methods.

	UPLC–MS/MS (ESI⁻, SPE extraction) all metabolites specific assay		UPLC–MS/MS (ESI⁻, SPE extraction) total diosmetin β-glucuronidase hydrolysis (*E. coli*)		HPLC–MS/MS (APCI⁺, LLE extraction) total diosmetin β-glucuronidase hydrolysis (*E. coli*)	
	Diosmetin (ng/ml)	3′-β-D-Diosmetin glucuronide (ng/ml)	Total diosmetin (ng/ml)	Equivalent 3′-β-D-diosmetin glucuronide (ng/ml)	Total diosmetin (ng/ml)	Equivalent 3′-β-D-diosmetin glucuronide (ng/ml)
Subject 4 Oh	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Subject 4 2h30	0.11	BLQ	BLQ	BLQ	BLQ	BLQ
Subject 4 4h	0.19	BLQ	BLQ	BLQ	BLQ	BLQ
Subject 4 12h	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Subject 4 24h	BLQ	BLQ	0.17	0.29	0.19	0.3
Subject 9 Oh	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Subject 9 7h	BLQ	BLQ	0.57	0.9	1.34	2.13
Subject 9 8h	BLQ	BLQ	1.61	2.55	1.98	3.14
Subject 9 12h	BLQ	16.0	7.7	12.0	8.1	12.9
Subject 9 24h	BLQ	BLQ	0.55	0.88	0.46	0.72
Subject 10 Oh	BLQ	BLQ	BLQ	BLQ	BLQ	BLQ
Subject 10 7h	BLQ	BLQ	0.16	0.25	0.19	0.29
Subject 10 8h	BLQ	BLQ	0.26	0.42	0.36	0.57
Subject 10 12h	BLQ	5.0	2.4	3.8	2.5	3.9
Subject 10 24h	BLQ	1.8	0.45	0.72	0.49	0.77
Detection limits (ng/ml)	0.1	1	0.1	0.2	0.1	0.2

thetic glucuronide metabolites or a total diosmetin assay involving hydrolysis with *E. coli* β-glucuronidase, free of interference, are totally concurrent with historical data. The full study as well as all related analytical methods are planned to be published in detail.

The HPLC–MS/MS assay described by Campanero et al. [1] using *Helix pomatia* as a source of hydrolytic enzymes is therefore biased and not adapted to the bio-analysis of diosmetin in human plasma samples. Consequently the analytical approach and its use in pharmacokinetic bioequivalence studies are not valid as well as the absence of coherence with data obtained for diosmetin after oral administration of Daflon® 500 mg.

References

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Response

There are a few concerns that I would like to point about the Letter to Editor of Walther and his group:

1. *Helix pomatia* β-glucoronidase has been used as a hydrolytic extract agent for the determination of the flavonoid aglycone diosmetin after oral administration to humans in three independent studies. The first work was published by Cova et al. [1] on 1992. Samples were incubated with 1250 β-glucoronidase units prior chromatographic analysis. In the second study plasma was incubated with 20 μl of β-glucoronidase/sulfatase solution Type H-2 [2]. In our work samples were incubated with 50 μl of diluted solution of β-glucoronidase/sulfatase Type H2-S. In all cases, no measured concentrations were observed in the chromatograms of extracts of hydrolysed flavonoid-free human plasma with a LOQs of 20, 2 and 0.25 ng/ml, respectively. As an example, Table 2 shows the chromatographic response observed in predose samples obtained before the administration of a single dose of micronised purified flavonoid fraction of diosmin (500 mg) to 32 healthy volunteers. In all the cases the chromatographic response is less than 20% of the observed for the LOQ. Therefore it can be considered that none interfering agents are present in the samples at diosmin retention time in accordance with the FDA [3] and EMEA [4] guidelines.
2. Otherwise Walther and his group have reported in his letter diosmin concentrations between 0.25 μg/ml to the μg-range after the analysis of two batch of β-glucoronidase/sulfatase solution Type H-2S. The equivalent concentrations after the application of our sample extraction procedure should be 5–25 ng/ml?, two values upper to the fixed LOQ. However, the chromatographic response observed in our work (see Table 2) after the chromatographic analysis of extracts of hydrolysed flavonoid-free human plasma samples obtained before drug administration is negligible. Similar data are reported by Spanakis et al. (see Fig. 2A of Ref. [2]). On the other hand, the reported data by Walther and his group have shown a great variability in diosmetin measure after the analysis of the different batches of *Helix pomatia* β-glucoronidase (0.25 μg/ml to the μg range). It should be throughly clarified and explained.
3. Pharmacokinetic results. The results obtained in our work are similar to the obtained in the only pharmacokinetics study that has been published to the date. Cova et al., reported Cmax values of 417 ± 94.1 ng/ml after administration of diosmin single oral dose of 10 mg/kg to healthy volunteers [1], whereas the values obtained in our study were 397 ± 202.5 ng/ml after oral administration of a single dose of Daflon® (approximately 7 mg/kg). However, the reported values by Walther and his group are erratic and inconsistent with the diosmin pharmacodynamic mechanism. Diosmin, as other flavonoid glycosides, is hydrolized by enzymes of the intestinal bacterial microflora into its aglycone, diosmetin. Next, it is subsequently absorbed into the systemic circulation. Therefore, diosmetin is the pharmacologically active form of this drug and relevant concentrations may be maintained along the time-dose to enhance venous microcirculation.
4. Phase II sulfonation also plays an important role in the metabolism of flavone aglicones [5,6]. For this reason we have used an enzyme with β-glucoronidase/sulphatase activity. How-

Table 2
Chromatographic response observed in pre-dose samples obtained from healthy volunteers before the administration of the micronised purified flavonoid fraction of diosmin.

Analytical batch	Volunteer	Sample IDENTIFICATION	Chromatographic area		% LOQ
			LOQ	Pre-dose sample	
1			24,070,178		
	1	1		2,645,220	10.99
	2	39		2,746,084	11.41
	3	77		2,233,675	9.28
2			2,668,309		
	4	115		172,488	6.46
	5	153		254,716	9.55
	6	191		479,288	17.96
3			1,541,462		
	7	229		151,921	9.86
	8	267		195,983	12.71
	9	305		298,888	19.39
4			10,911,649		
	10	343		1,967,254	18.03
	11	381		1,080,658	9.90
	12	419		2,027,331	18.58
5			3,162,019		
	13	457		364,252	11.52
	14	533		128,438	4.06
	15	495		555,326	17.56
6			1,647,563		
	16	571		111,185	6.75
	17	609		220,100	13.36
	18	647		315,898	19.17
7			929,337		
	19	685		22,101	2.38
	20	723		61,877	6.66
	21	761		59,799	6.43
8			1,390,485		
	22	799		158,701	11.41
	23	837		19,617	1.41
	24	875		83,155	5.98
9			1,827,570		
	25	913		129,692	7.10
	26	951		120,909	6.62
	27	989		190,776	10.44
10			654,369		
	28	1027		89,839	13.73
	29	1065		19,061	2.91
	30	1103		13,019	1.99
11			297,595		
	31	1141		32,561	10.94
	32	1179		49,055	16.48

ever, the enzyme used by Walther and his gruop (*Escherichia coli* β-glucoronidase) only showed β-glucoronidase activity (see Sigma–Aldrich product information of β-glucoronidase from *Escherichia coli*). Therefore, seems to be an inappropriate choice to evaluate pharmacokinetic profile of total diosmetin after enzymatic hydrolysis.

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